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WHOLE-BODY PROTEIN TURNOVER AND ENERGY METABOLISM IN MAN

A thesis submitted to the University of London for the degree of
Doctor of Philosophy in the Faculty of Medicine

by

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ABSTRACT

This thesis consists of 2 major parts - Part A on whole-body protein turnover in humans, and Part B on energy metabolism.

The method primarily used for measurement of whole-body protein turnover in this thesis has been with the constant infusion of (1-¹⁴C)leucine, and this method is tested, analysed, and developed for extended 24 - 40 hour continuous protein turnover measurements. Using this method, it was found that protein intake has a dramatic and controlling influence on protein synthesis. This is initially established in Section 3.

In Section 4, it is shown that protein synthesis, breakdown, and oxidation are acutely responsive to protein intake, with large diurnal fluctuations resulting from feeding/fasting patterns. Protein turnover is compared in obese and lean humans, amino acid pool changes and the question of circadian rhythms examined.

Section 5 compares results from the (1-¹⁴C)leucine method with those of (¹⁵N)glycine, and long term changes in protein turnover are monitored. Section 6 shows how remarkable adaptive changes occur in protein synthesis and breakdown, resulting from a prolonged absence of protein intake.

Thus the relationship between whole-body protein turnover and protein intake is examined and established.

In Part B, techniques developed for the (1-¹⁴C)leucine method are exploited to measure by indirect calorimetry acute changes in the net synthesis and utilisation of glucose and fat. It is suggested that traditional methodology and restrictive current concepts concerning the use of indirect calorimetry have limited its development. Formulae

are derived, which allow calculation of net fuel transfers under most conditions, and these formulae and (1-¹⁴C)leucine techniques are combined to measure the body's response to different levels of carbohydrate intake, in both obese and non-obese humans.

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I wish to express grateful appreciation to my supervisor, Professor J.C. Waterlow. His inspiration as a great scientist, and his encouragement of both freedom of thought and research, have greatly influenced my own research capability. Likewise I am greatly appreciative of the friendship, guidance, and encouragement I received from Peter Garlick.

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..... thanks a million for the typing Fiona (Hibbett).

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PART A

WHOLE-BODY PROTEIN TURNOVER

SECTION 1 INTRODUCTION

1.1. WHOLE-BODY PROTEIN TURNOVER AND ITS MEASUREMENT

1.2. ESTIMATION OF FLUX FROM MEASUREMENTS ON PLASMA

1.3. BACKGROUND TO THE PRESENT USE OF (1-¹⁴C)LEUCINE
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1.4. ESTIMATION OF FLUX BY EXCRETED END-PRODUCT

1.5. BACKGROUND TO THE PRESENT USE OF (¹⁵N)GLYCINE

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1.1. WHOLE-BODY PROTEIN TURNOVER AND ITS MEASUREMENT

Two basic aims underlie the work in this thesis - the development of a specific method for estimating whole-body protein turnover in humans, and the measurement of absolute values and patterns of change in whole body protein synthesis and breakdown.

The concept that all body proteins are in a dynamic state of turnover, with their own individual patterns of continuous breakdown and renewal, has only been recognised in the last few decades, and indicates even further the awesome complexity of protein metabolism. Nitrogen balance studies have been the traditional method for assessing the state of whole-body protein metabolism in man, measuring differences between nitrogen intake and excretion under different conditions of health and dietary intake, and then attempting to deduce the metabolic significance in terms of net protein anabolism or catabolism. Useful as this method has been, it can only measure the end-result and net balance between whole-body protein synthesis and breakdown, yet it is the dynamics of these protein turnover processes that are ultimately responsible for growth in children, the wasting seen in malnutrition and disease states, and changes in lean body mass resulting from a wide spectrum of dietary conditions. Therefore the recent development of methods for measurement of whole-body protein synthesis and breakdown offers the opportunity for a 'great leap forwards' in our understanding of human protein metabolism.

These methods involve the administration of isotopically labelled amino acids. In animals this has meant the ability to measure protein synthesis and breakdown in most tissues and under many different conditions, essentially by measuring the rate of incorporation of label into the protein of a particular tissue to determine its synthesis rate, or by

measuring its subsequent rate of loss from the tissue to determine both breakdown and synthesis.

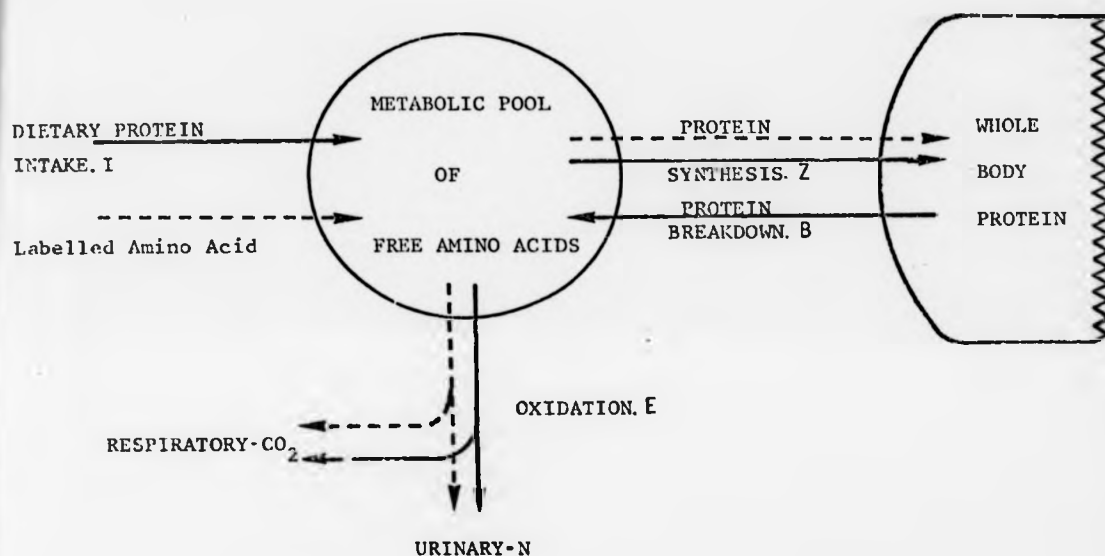
In man, because it is rarely possible to obtain tissue samples, efforts have largely concentrated on the measurement of whole-body protein turnover. This quantity is comprised of the sum of turnover rates of all individual body proteins, and Waterlow (1969) has pointed out the analogy with basal metabolic rate, which is the sum of the oxygen turnover rates of all cells in the body.

Whole-body protein turnover research can be seen to have developed along two distinct but related paths - the refinement of measurement techniques, and investigation of actual rates of whole body protein synthesis and breakdown especially in relation to growth, malnutrition and pathological conditions. In a very miniaturised way, the work in this thesis involves both those pathways. The underlying principles of whole body protein turnover and its measurement are outlined below, particularly those applicable to the methods used in this thesis.

Since the first attempt to obtain quantitative measurements of whole-body protein turnover in man (Sprinson & Rittenberg, 1949) using a single dose of (^{15}N)glycine, much research has been put into developing methods and techniques that might improve accuracy, precision and validity of protein turnover measurements. This has led to the use of amino acids with stable or radioactive labelling, different methods of sample collection, and often very complicated analysis models derived from alternative approaches to the calculation of results.

The simplest model representing whole body protein metabolism, used for calculating whole body protein synthesis and breakdown when labelled amino acids are given, is shown in Figure 1.

FIGURE 1.1. The 2-pool model



The underlying assumptions in the use of this model are:

- (i) the synthesis and breakdown of all body proteins occur from one homogeneous precursor pool (the 'metabolic pool');
- (ii) that a labelled amino acid introduced into that pool mixes completely and reflects the metabolism of all amino acids;
- (iii) that protein synthesis and amino acid oxidation are the only significant pathways out of the metabolic pool, and protein breakdown and dietary intake the only significant pathways in;

- (iv) that while measurements are being made the pool size remains constant so that -

$$Z + E = I + B = Q \text{ ("flux")};$$

- (v) that no recycling of labelled amino acid occurs from the protein pool.

All these assumptions introduce some errors, and different measurement and calculation techniques have attempted to minimise these.

The aim of most methods of measurement of whole-body protein turnover is to estimate flux (Q), the rate of total amino acid flow through the free amino acid metabolic pool. However, there are two basically different approaches in the methods which attempt to estimate flux:

- (1) Measurements on plasma.
- (2) Measurements on excreted end-products.

Both approaches involve their own set of assumptions, error sources, advantages and disadvantages, but nevertheless flux can be calculated from the same fundamental equation (Equation 1).

When a labelled amino acid (eg. ^{14}C - or ^{15}N - labelled) is introduced at a constant rate (i) into the metabolic pool, the specific activity of the free amino acid (or relative abundance of ^{15}N) rises to a constant plateau value (S). The flux (Q) is then calculated from an equation which reflects the fact that the amount of isotope entering the metabolic pool (ie. by infusion) must equal the amount leaving.

$$i = Q.S$$

Equation 1

Both plasma and end-product approaches attempt to measure the isotope concentration term (S), assuming that this is a reasonable estimate of specific activity in the precursor pool for protein synthesis. This allows the calculation of Q.

It is in the use of the model's oxidation path that the two approaches show a basic difference. In methods based on the plasma measurement of flux, this pathway is used to determine only the oxidation rate (E). In excreted end-product methods, the products of the oxidative pathway are used to determine both flux (Q) and protein oxidation (E). However, despite this difference, both approaches use the same oxidative pathway equation to calculate these quantities, based on the assumption that the proportion (e) of tracer dose or infusion (i) being channeled into oxidation, reflects the proportion of flux (Q) being channeled into oxidation (E). Put another way, the proportion of oxidation end-product that is labelled (e/E) reflects the proportion of labelling in the metabolic pool, and hence reflects the proportion of flux that is labelled (i/Q). Thus Equation 2

$$e/i = E/Q \quad \text{or} \quad e/E = i/Q \quad \text{Equation 2.}$$

Plasma measurement methods use this relationship to calculate E, as flux (Q) has already been measured from plasma specific activity. For excreted end-product methods, Equation 2 is equivalent to Equation 1 because the proportion of labelling of the end-product (e/E) is assumed to represent the specific activity (S) in the precursor pool, and is thus used to calculate flux (Q).

Both approaches then use Equation 3, previously shown as one of the model's assumptions, to calculate whole-body protein synthesis (Z); and protein breakdown (B) where the intake of protein (I) is also known.

$$Q = Z + E = I + B \quad \text{Equation 3.}$$

With both the plasma measurement and excreted end-product methodologies, there are two different isotope administration techniques - the single dose and continuous administration (eg. infusion), each of which has advantages and limitations. And for all four of these

techniques there are 2 alternative analysis models that can be used to calculate a value as an estimate of flux - compartmental analysis and stochastic analysis. However compartmental analysis can only be applied to rising or falling specific activity-time curves, and is consequently of little value when constant infusion methods are used. In their book *Protein Turnover in Mammalian Tissues and in the Whole Body*, Waterlow, Garlick and Millward (1978) have discussed in detail the techniques that have been used to estimate flux and calculate whole-body protein turnover.

Two methods have been used for the protein turnover data presented in this thesis. They include:

- (1) the constant infusion of (1-¹⁴C)leucine with measurements of plasma leucine specific activity to estimate flux;
- (2) the (¹⁵N)glycine method, administered both continuously or by single dose, with measurements of the abundance of ¹⁵N in excreted urinary ammonia, to estimate flux.

However, this thesis is primarily orientated around the use of (1-¹⁴C)leucine - a plasma measurement method. One of the underlying aims of the work has been to develop this method, test its limitations and extend its use. The (¹⁵N)glycine method has been used for both comparative purposes, and for carrying out some of the repeated measurement monitoring of protein turnover.

The following discussion briefly summarises some of the main assumptions and limitations specific to each method for estimating flux, and particularly as they apply to the isotopes and techniques used.

1.2. ESTIMATION OF FLUX FROM MEASUREMENTS ON PLASMA

The underlying assumption, with plasma measurements of specific activity, in addition to those already listed for the two pool model, is that the plasma represents the precursor pool from which protein synthesis occurs. In fact the nature and boundaries of the true precursor pool remain obscure for both tissues and whole body. How it relates to the intracellular free amino acid pool or the plasma pool is not understood, nor whether the precursor pool for protein synthesis is immediately related to the free amino acids derived from protein breakdown. Perhaps the true precursor pool cannot be defined for the whole body, nor directly sampled, as its functional boundaries may differ in different tissues and it may change under varying conditions. However evidence suggests that it lies between the intracellular free amino acid pool and plasma free amino acid pool, and the closer functionally it relates to the plasma pool, the better will plasma specific activities reflect precursor pool specific activities.

Nevertheless estimates of flux from plasma measurements are likely to be too low. This is because the values that are actually calculated from plasma specific activity measurements, whether by stochastic or compartmental analysis, are usually "disposal rates" of amino acid from the plasma pool (i.e. its net rate of disappearance from the plasma). This does not take account of internal recycling in which amino acids released by protein degradation enter and dilute the precursor pool and are then immediately available for protein synthesis again, without entering the plasma pool. Clearly the specific activity of a tracer introduced into the plasma amino acid pool will be higher than its specific activity in the precursor pool which cannot unfortunately be

directly sampled, and consequently estimates of precursor pool flux, derived from plasma disposal rate measurements, will be too low.

The mathematical principles underlying the calculation of plasma amino acid disposal rate relate to two different analytic approaches - compartmental analysis and stochastic analysis. However both methods turn out to be mathematically equivalent if used to calculate disposal rates using the two pool model.

Compartmental analysis can only be applied to rising or falling specific activity-time curves in plasma (or urine), so that although it can be applied to the rise to plasma plateau in a constant infusion, there is often difficulty in accurately defining that early part of the curve. It is more appropriate to use the falling specific activity-time curves in plasma following single dose tracer administration.

In compartmental analysis a multi-exponential equation is fitted to the falling specific activity curve. It assumes that the change in plasma specific activity results from the flow of tracer through a series of compartments in steady state, with first order kinetic exchange between them, and with each exponential term representing one compartment. By curve peeling or computer analysis usually 3 exponential terms have been derived although Heath & Barton (1973) obtained 4. Waterlow, Garlick & Millward (1978) point out that the apparent existence of multi-compartments is probably not only unverifiable, but in fact may not represent reality, whilst factors such as measurement error and biological variation significantly contribute to the curve form. The general multi-exponential equation which is fitted to a plasma specific activity curve is:

$$S_t = X_1 e^{-\lambda_1 t} + X_2 e^{-\lambda_2 t} + \dots + X_n e^{-\lambda_n t} = \sum_{i=1}^n X_i e^{-\lambda_i t}$$

Equation 4.

where S_t is the plasma specific activity as a function of time t , and $X_{1...n}$ are exponential coefficients, $\lambda_{1...n}$ exponential rate constants and n the number of compartments. The techniques of curve fitting and mathematics of curve peeling, and solution of the multi-exponential equation for the exponential parameters are given in detail by Atkins (1969) and Shipley & Clark (1972).

The fitted exponential equation can be used to calculate estimates of flux in a number of ways. These include laboriously calculating a value for the absolute transfer rate from a theoretical compartment that may represent the precursor pool, or most simply, calculating the plasma amino acid disposal rate \dot{D} , known to underestimate flux by not accounting for internal recycling. The disposal rate \dot{D} is the easiest to calculate, using the equation

$$\dot{D} = \frac{d^*}{\sum_{i=1}^n \frac{X_i}{\lambda_i}} \quad \text{Equation 5.}$$

where d^* is the dose of tracer, and the terms X_i and λ_i the derived parameters from the appropriate multi-exponential equation. In addition the order of error associated with internal recycling can at least be approximated from other methods, such as comparison between oxidation rates measured by $^{14}\text{CO}_2$ and by urinary N outputs, or by tissue intracellular pool measurements in animals. Therefore, disposal rate \dot{D} is used as the best and most convenient estimate for flux.

The substantial disadvantages associated with compartmental analysis include its being of little value for constant infusions, the complicated calculations involved, its dependence on values derived from exponential curve fitting and therefore possible errors, and the probably unverifiable assumptions it requires about the system.

Stochastic analysis, in distinct contrast, can be used with plasma specific activity measurements from both single dose or continuous infusion methods, it makes no assumptions about compartments, it uses direct measurements, and the calculations are simple. It calculates the plasma disposal rate, and so as an estimate of flux, is probably too low because of internal recycling.

When a tracer amino acid has been given as a single dose (d^*), and the falling plasma specific activity values (S_t) produce the characteristic specific activity-time curve, once the whole dose (d^*) has cleared the plasma pool then

$$d^* = \dot{D} \int_0^{\infty} S_t dt \quad \text{Equation 6}$$

where \dot{D} is the plasma amino acid disposal rate. This can simply be shown to be mathematically equivalent to Equation 4 from compartmental analysis.

For the single dose technique the area under the plasma specific activity-time curve needs to be evaluated to calculate \dot{D} . When the labelled amino acid is administered by constant infusion at rate (i), the plasma specific activity rises to a constant plateau (S) so that rate of tracer entry in to the plasma pool is equal to the net amount leaving, and thus at plateau

$$i = \dot{D} \cdot S \quad \text{Equation 7}$$

This is analogous to Equation 1, except that it indicates that plasma amino acid disposal rate is used as an estimate of flux.

A comparison of the relative merits of administration of labelled amino acid by single dose or by constant infusion, substantially favours the constant infusion method. The major advantage of this method is

the ease and accuracy of specific activity definition at plateau, and hence the better estimate of D. Furthermore, fewer blood samples are needed to define the plateau value than are needed for definition of the specific activity curve following a single dose. Constant infusion also avoids the error that may arise at the tail end of the single dose specific activity curve, caused either by the need to extrapolate or by the necessity to distinguish when recycling of label is significantly contributing to the tail.

Recycling of label also occurs at a very slow rate with constant infusions so that the 'plateau' specific activity is really a gradually rising 'pseudo-plateau', but in infusions of short duration this is probably negligible. This effect can be seen in figures in Sections 4 and 6.

With measurements on plasma for estimates of flux, and thereby protein turnover, one further source of error is the conversion of amino acid flux into protein turnover, the conversion factor being an estimate of the weighted mean of the concentration of the particular tracer amino acid in all body proteins.

The particular tracer amino acid used for the plasma measurement method of estimating protein turnover, has been (1-¹⁴C)leucine. The reason for its choice, and the background studies that led up to its use, are outlined in the following resumé.

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The particular tracer amino acid used for the plasma measurement method of estimating protein turnover, has been (1-¹⁴C)leucine. The reason for its choice, and the background studies that led up to its use, are outlined in the following résumé.

1.3. BACKGROUND TO THE PRESENT USE OF THE (1-¹⁴C)LEUCINE CONSTANT INFUSION METHOD.

Measurements of plasma specific activity to estimate flux have usually implied the use of radioactive amino acid tracers, at least until the very recent use of the stable (¹³C)leucine. One of the major advantages of radioactive tracers is the sensitivity and ease of measuring their specific activities in plasma or tissues, and hence the development of the plasma measurement method.

When first used to measure whole body protein turnover, radioactive amino acids were given as a single dose either intravenously or orally. (³⁵S)Methionine (Maurer, 1960), (¹⁴C)phenylalanine (Grumer *et al*, 1962) and (³H)glycine (Nyhan & Childs, 1964) were used, but assumptions about parts of the specific activity-time curves and calculations amounting to compartmental analysis resulted in protein turnover rates that were not correct.

Waterlow (1967) was the first to use the constant infusion technique to measure whole body protein turnover in man, using uniformly labelled (U-¹⁴C)lysine. A problem that then became apparent was the inconveniently long (10 - 18 hours) rise to plateau, resulting from the large size of the plasma free lysine pool (166 μ moles/litre, Möller *et al*, 1979). Uniformly labelled (U-¹⁴C)tyrosine was subsequently chosen (James *et al*, 1976) because of its much smaller plasma free tyrosine pool (60 μ moles/litre) producing a more rapid rise to plateau (about 4 hours), and because a convenient automated method had been developed for its measurement.

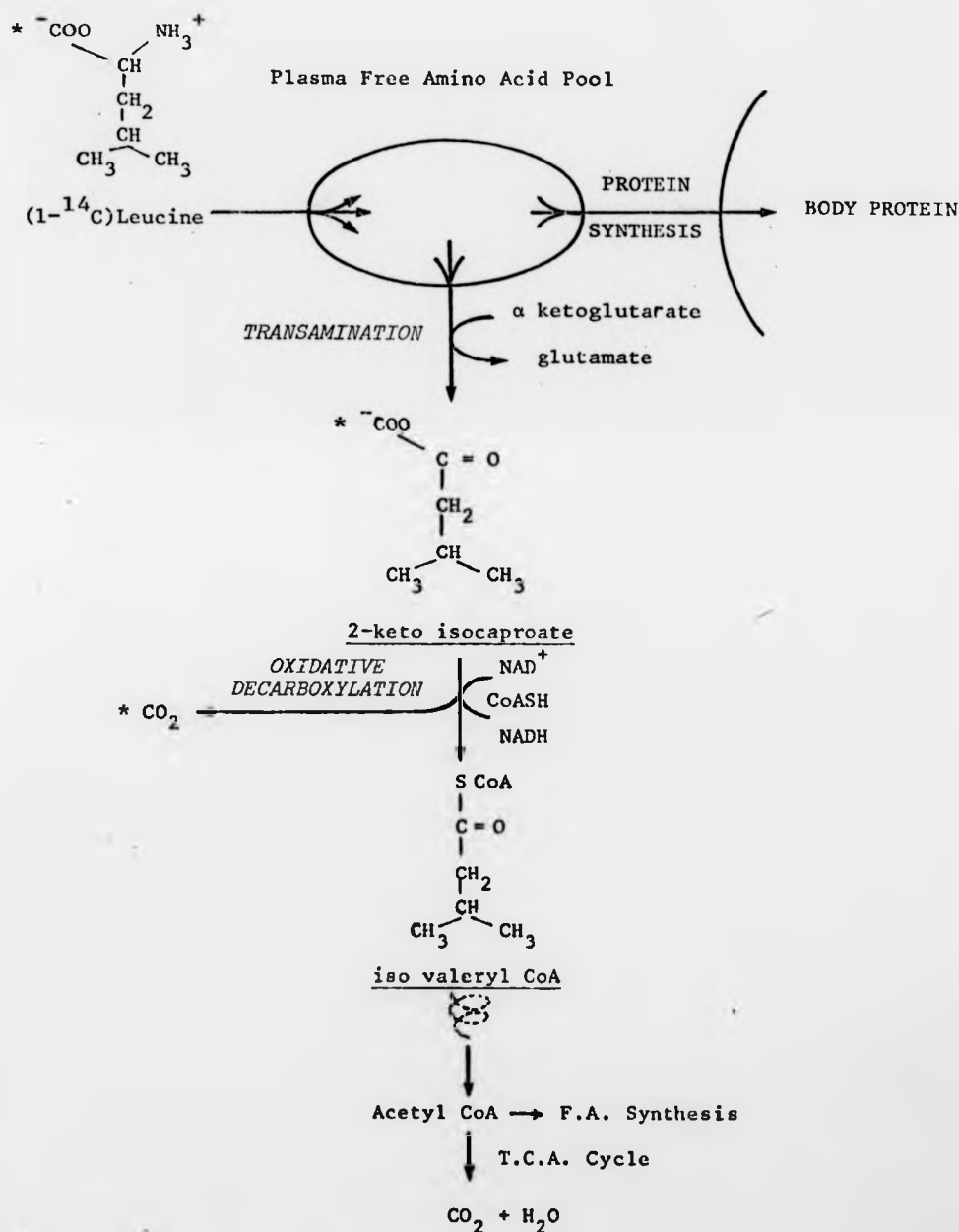
With the early infusions of (U-¹⁴C)lysine and (U-¹⁴C)tyrosine, the estimated flux rate was taken to approximate the rate of whole body protein synthesis. The oxidation rate was assumed to be small compared

with synthesis, and it was felt that the resultant small overestimate of synthesis would be partially balanced by the underestimate arising from internal recycling. However it was found with (U-¹⁴C)tyrosine that amino acid oxidation could be measured, because the proportion of infused radioactivity expired in CO₂, represented the proportion of flux being oxidised (James et al, 1974; Sender et al, 1975).

Unfortunately, problems emerged with (U-¹⁴C)tyrosine when the expired CO₂ specific activity sometimes failed to reach plateau even after 10 hours, despite achievement of a rapid plasma plateau. This was probably because some of the labelled carbon from the (U-¹⁴C)tyrosine skeleton (as with any uniformly labelled amino acid) would not have been immediately excreted as CO₂, but recycled into fatty acid synthesis or gluconeogenic pathways, before eventually being excreted. In addition tyrosine is non-essential.

(1-¹⁴C)Leucine was consequently introduced (O'Keefe et al, 1974), as it seemed likely to overcome all the above problems. It seemed to meet all the major theoretical requirements for a tracer amino acid to succeed in measuring whole body protein turnover from plasma measurements. It is an essential amino acid so there is no *de novo* synthesis. It has no known significant metabolic pathways other than oxidation and incorporation into protein synthesis. It has a reasonably small plasma free leucine pool (121 μ moles/litre), and reaches plasma plateau in a conveniently short period (5 - 8 hours). In its oxidative pathway, immediately following the first step (transamination), irreversible oxidative decarboxylation occurs (Figure 1.2), removing the ¹⁴C as ¹⁴CO₂ which is then expired, so that after applying a known correction factor to account for a loss of some 10% of the ¹⁴CO₂ into bone and other pathways, expired ¹⁴CO₂ is an excellent measure of leucine oxidation, and specific activity of expired CO₂ reaches plateau

FIGURE 1.2. Leucine metabolism.



within about 1 hour of acquiring plasma plateau.

By comparing oxidation rates derived from the (1-¹⁴C)leucine method with those from urinary N excretion, Golden & Waterlow (1977) calculated that rates of flux and protein synthesis based on plasma measurements underestimated precursor pool flux and true protein synthesis by 20%, and that the leucine specific activity at the site of oxidation (and thus supposedly protein synthesis) was only 80% of that in the plasma. Internal recycling of leucine from protein degradation into the precursor pool was thought to have been responsible for this. Thus their figures imply that 20% of amino acids entering the precursor pool come from protein breakdown, and 80% from plasma. This factor is considered further in this thesis.

It can be seen that there are several major advantages in the techniques that have been developed for estimating whole body protein turnover from plasma specific activity measurements.

- (1) Radioactive tracers, used most commonly with this technique, are easily and accurately measured, although stable isotopes (¹³C and ¹⁵N) can now be measured in plasma.
- (2) Constant infusion gives clearly and accurately definable plateau values for plasma specific activity, from which amino acid disposal rate is calculated and flux estimated. This is more accurate and requires fewer blood samples than a single dose technique.
- (3) (1-¹⁴C)Leucine is an excellent tracer amino acid, being an essential amino acid, with no known significant metabolic pathways other than oxidation and protein synthesis, and having a rapid rise to plasma specific activity plateau immediately followed by a specific activity plateau for expired CO₂.

- (4) Oxidation rate of leucine, as a proportion of flux, can be directly measured from $^{14}\text{CO}_2$ excretion. This can be combined with leucine intake and nitrogen balance to estimate internal recycling, which can then be accounted for in the estimation of protein synthesis rates.

It was against this background that the work for this thesis commenced in 1975, soon after the introduction of (l- ^{14}C)leucine as a tracer for protein metabolism (O'Keefe et al, 1974). As indicated earlier, one of the aims has been to take up and develop the use of the (l- ^{14}C)leucine constant infusion method, and by infusing large numbers of subjects (well over 1,200 hours of infusion) establish consistent values for adult humans under various dietary conditions.

1.4. ESTIMATION OF FLUX BY EXCRETED END PRODUCT

With both the plasma measurement method, and the excreted end product method, the underlying aims are identical, i.e. to estimate the specific activity (relative abundance) of tracer or isotope in the precursor pool, so that the same set of general equations (Equations 1 - 3) can then be applied, and protein synthesis and breakdown rates calculated. The previous section has shown that plasma specific activity measurements of amino acids differ significantly from precursor pool specific activities, and the assumptions on which the plasma method is based, particularly in assuming a non-compartmented homogeneous precursor pool which ignores internal recycling, can lead to significant (though partially quantifiable) error.

Similarly there are assumptions of doubtful validity underlying the use of the excreted end-product method to estimate precursor pool flux.

The use of urinary end products for determining whole body protein turnover, implies that the tracer is ^{15}N . The method assumes that if an amino acid labelled with ^{15}N is given, it will enter the metabolic pool and reversibly transfer the ^{15}N label with other amino acids in such a way that ^{15}N metabolism represents total amino N metabolism. It is further assumed that this metabolic pool is the precursor pool for both protein synthesis and amino acid oxidation, and that the N-containing end-product excreted in the urine is derived from the same precursor pool as protein synthesis, and only from that pool. Thus the specific activity of the end-product N is assumed to represent the specific activity of amino acid N in the precursor pool. Hence measurement of the relative abundance of ^{15}N in the chosen urinary end-product (eg. NH_3), should represent ^{15}N relative abundance in the precursor pool.

Although both compartmental and stochastic analysis can be used to estimate flux by calculating disposal rate, the same complex difficulties arise with compartmental analysis as discussed with its use for plasma specific activity measurements. Olesen *et al* (1954) fitted a 3-exponential term equation to the 15 day curve of urinary excretion of ^{15}N after a single dose of (^{15}N)glycine, and by assuming some re-entry limitations proposed a 4-pool model. Compartmental analysis was then used to determine exponential parameters, rate constants and pool sizes and hence an estimate of flux.

In contrast, stochastic analysis is unconcerned with the number or size of protein pools or excretory pools, but calculation of realistic disposal rates from the metabolic pool greatly depends on the validity of the assumptions concerning both the tracer, and the derivation of end product from (and only from) the metabolic pool, as outlined above. If these assumptions are valid, then the proportion of the given dose of ^{15}N that is excreted in the chosen end product would be the same as the proportion of amino acid disposal which is excreted as the end product.

Consequently, if the chosen urinary end product is being excreted at rate (\dot{E}), and an ^{15}N -labelled amino acid is given by constant infusion at rate (i) so that the rate of excretion of ^{15}N in the chosen end product rises to a constant rate (\dot{e}), then

$$\dot{e}/i = \dot{E}/D \quad \text{Equation 8}$$

where D is the amino acid disposal rate. Since at plateau the relative abundance (S) of ^{15}N in the end product is \dot{e}/\dot{E} then Equation 8 becomes:

$$i = D \frac{\dot{e}}{\dot{E}} = D.S \quad \text{Equation 9}$$

which is identical to stochastic analysis equations for measurements on plasma specific activities (Equation 7).

When an ^{15}N amino acid is given as a single dose (d) and its ultimate *cumulative* excretion in the chosen urinary end product is (e_c), then on the stochastic principle that the proportion of tracer dose excreted in the end product is equal to the proportion of amino acid disposal rate being excreted as the end product,

$$e_c/d = \dot{E}/\dot{D} \quad \text{Equation 10}$$

The single dose method requires calculation of the area under the relative abundance - time curve to achieve a value of the ultimate cumulative abundance of ^{15}N in the chosen end product

$$(\text{i.e. } \int_0^{\infty} S \, dt = e_c/\dot{E}). \text{ In practice this area is approximated}$$

by summation of values of isotope abundance (S_t) from a consecutive series of urine collections at times intervals ($t=0, t_1 \dots T$) so that Equation 10 becomes

$$\dot{D} = d/e_c/\dot{E} = d/\int_0^T S_t \, dt = d/\sum_{t=0}^T (S_t \cdot T) \quad \text{Equation 11}$$

There is real difficulty in defining the time point T , when collection should cease because isotope clearance is sufficiently complete, but before isotope re-entry from the protein can significantly affect the tail of the curve. Much of the comparative work between (^{14}C)leucine and (^{15}N)glycine (although not presented in this thesis) was aimed at clarifying this methodological problem.

The problems faced in deciding which ^{15}N labelled amino acid, and which urinary end product to choose, reflect some of the difficulties presented by the underlying assumptions of this method.

The basic requirement for any ^{15}N labelled tracer is that the ^{15}N should be re-distributed in such a way that it represents the metabolism of all amino-N. Since it is most unlikely that all precursor pool amino acids become equally labelled, the essential requirement is for the same proportions of different labelled amino acids to enter protein synthesis as enter oxidation. In fact this cannot be fully satisfied as the majority of body protein synthesis is separate from the liver where a substantial proportion of oxidation and hence urea synthesis occurs.

This is also the objection to the other main assumption of ^{15}N methods - that the same precursor pool of amino acids serves protein synthesis, and whole body amino acid oxidation with its resultant end product formation. Obviously this cannot be true, as urea is only formed in the liver, urinary ammonia is mainly formed from glutamine in muscle so that the precursor pools for both these end products are unlikely to be representative of a 'whole body' precursor pool.

An advantage of the (1- ^{14}C)leucine method is that although errors are associated with its underlying assumptions, they can largely be accounted for and even quantitated. With the ^{15}N excreted end-product method, the errors associated with assuming a common synthesis/end-product precursor pool cannot be tested as there is no such pool.

Because of the difficulties associated with these basic assumptions, two questions of great practical importance remain:

- (1) which ^{15}N labelled amino acid gives the most valid protein turnover measurement?

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An advantage of the (1- ^{14}C)leucine method is that although errors are associated with its underlying assumptions, they can largely be accounted for and even quantitated. With the ^{15}N excreted end-product method, the errors associated with assuming a common synthesis/end-product precursor pool cannot be tested as there is no such pool.

Because of the difficulties associated with these basic assumptions, two questions of great practical importance remain:

- (1) which ^{15}N labelled amino acid gives the most valid protein turnover measurement?

- (2) which urinary end-product should be used (bearing in mind the tracer amino acid used), to give a relative abundance of ^{15}N most representative of the precursor pool?

A third practical question with its own set of possible errors has also been tackled in the development of this method, and concerns the simplicity and convenience of the method:

- (3) which method of tracer administration (eg. single dose, multiple dose, constant infusion, etc) gives satisfactory results with the greatest convenience?

The following resumé of previous use of ^{15}N /excreted end-product method summarises how these questions have been approached in the past, and how they relate to some of the ongoing programmes, of relevance to this thesis. One of the best available tests of the validity of the ^{15}N methods used, is a comparison of their estimates of absolute rates of whole body protein turnover and their ability to measure change, with other independent methods, such as (1- ^{14}C)leucine. Section 5 undertakes such a comparison of (1- ^{14}C)leucine against various ^{15}N methods.

1.5. BACKGROUND TO THE PRESENT USE OF (^{15}N) GLYCINE

The first attempt to measure whole body protein synthesis in man used oral (^{15}N)glycine, and was based on the simple 2 pool model (Sprinson & Rittenberg, 1949). Protein synthesis was calculated from the slope of the ^{15}N excretion curve. Subsequent studies tended to propose increasingly more complicated multi-compartmental models attempting to explain and solve the observed multi-exponential profiles of urinary ^{15}N excretion and thus achieve more likely estimates of protein synthesis (eg. San Pietro & Rittenberg, 1953; Olesen et al, 1954).

The early studies using ^{15}N produced rather scattered results and non-conclusive protein turnover changes in pathological states, and probably this uncertainty, along with the enormously complicated mathematics of these models, caused stable isotope protein turnover studies to fall from favour. More recent studies have tended towards simple models with simple analysis methods, with emphasis on making the technique a useful practical one that can give consistent sensible results. Picou & Taylor-Roberts (1969) developed the constant administration technique with (^{15}N)glycine, measuring plateau abundance of ^{15}N in urinary urea, and they used this method to measure changes in protein turnover in malnourished, recovering and recovered children. A disadvantage of this method was the relatively long times needed for the ^{15}N abundance in urea to reach plateau - 24 hours in children and 48 hours or longer in adults - a result of the large urea pool with its half-life of about 10 hours. The rates they measured were therefore *averages of whole body protein turnover over long periods*, this method being unable to show acute changes.

In theory any urinary N end product can be used to calculate flux, provided it is derived from the same 'metabolic pool' from which whole body protein synthesis occurs (and only from that pool), so that it reflects ^{15}N abundance from that pool. As previously mentioned, urea does not satisfy this criterion because it is synthesised in the liver, separate from synthesis of the majority of body protein, and so when (^{15}N)glycine is introduced and its ^{15}N transfers to other amino acids throughout the body, the proportions of different (^{15}N)amino acids entering protein synthesis are unlikely to be the same proportions oxidised and excreted as urea.

Recently the use of urinary ammonia has been suggested as a suitable end-product of N metabolism, its advantage being a more rapid rise to plateau of ^{15}N abundance in about 12 hours, during constant infusion with (^{15}N)glycine (Waterlow *et al*, 1978; see also Figure 2.5 in Section 2). Urinary NH_3 arises from different precursors to urea, the most important being the amide-N of glutamine (mainly from muscle), and also from direct metabolism of amino acids by the kidney, but in any case there appears to be no significant pool to delay its excretion. Some early comparative work has been done with (^{15}N)glycine and ($1\text{-}^{14}\text{C}$)leucine, estimating whole body protein synthesis from both urea and ammonia as end-product (Waterlow *et al*, 1978), and also simultaneously with ($1\text{-}^{14}\text{C}$)leucine (Golden & Waterlow, 1977). In malnourished and recovering children, the ammonia method persistently gave lower synthesis rates than either total N or urea methods, but all methods showed the same proportional effects. In elderly subjects the same relationship was also shown, and protein synthesis rates from ($1\text{-}^{14}\text{C}$)leucine were intermediate between the NH_3 and urea methods' values.

An ongoing programme directed by Professor J C Waterlow has been aimed towards further validating the NH_3 end-product method and simplifying the technique of dose administration. This programme conveniently coincided with the aims and work in this thesis, so that the testing of different techniques of the (^{15}N)glycine method, their ability to estimate whole body protein turnover could be compared with the (1- ^{14}C)leucine method - an advantage to both methods - as well as these techniques being used to monitor protein synthesis changes when the use of a radioactive tracer was precluded. This work is presented in Section 5.

1.6. PROTEIN TURNOVER AND DIET: BACKGROUND STUDIES

Several attempts have been made in the past to discover how levels of protein and energy intake, both long and short term, might affect protein synthesis and breakdown. However the results have been quite confusing.

Waterlow & Stephen (1967; 1968) using (U-¹⁴C)lysine in rats, showed a 30% decrease in flux following 6 weeks of protein depletion, but little change in flux following a variety of short term dietary changes. Tschudy et al (1959) measured protein turnover in a single subject with (¹⁵N)aspartate at 4 different combinations of protein and energy intake between 1200 Kcal/40 g protein and 1800 Kcal/80 g protein. They found increasing rates of synthesis with the increasing protein/energy intakes, but all their synthesis results were very low and probably inaccurate because of their use of the compartmental method of San Pietro & Rittenberg (1953). Kassenaar et al (1960) using the same method found no significant changes in synthesis rate in a patient with anorexia nervosa, fed at 5 different levels of protein and energy intake.

There was also no change in the rate of protein synthesis when children (recovered from malnutrition) were given different levels of protein intake at equal energy intake levels. However, an increased retention of N (ie. growth) was noticed on the higher protein diets, and the authors concluded that this resulted from a decrease in protein breakdown (Picou & Taylor-Roberts, 1969; Golden et al, 1977). However malnourished children on a maintenance diet prior to recovery were shown to have markedly decreased whole body protein synthesis rates, then during recovery synthesis rates rose to almost twice the 'normal' recovered rates (Golden et al, 1977). Nutritional status therefore appears to effect whole body protein synthesis rates.

In contrast to all these studies Sender et al (1975) demonstrated a 50% fall in whole body protein synthesis and a 60% fall in protein oxidation when 5 obese adults were measured by continuous infusion with (U-¹⁴C)tyrosine initially on a 2000 Kcal/70 g protein diet and then again after 3 weeks on 300 Kcal/0 g protein diet.

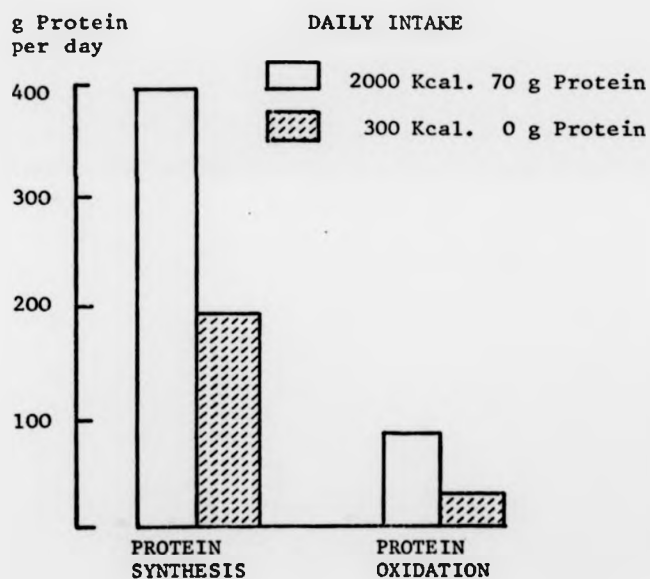


FIGURE 1.3. The effect on whole body protein synthesis and oxidation in 5 obese subjects, before and after 3 weeks on 300 Kcal protein-free diet. (From Sender et al, 1975).

1.7. SCHEME OF INVESTIGATIONS FOR THIS STUDY

The work presented in this thesis really had its beginning at this point in 1975, stimulated by the recent availability of (1-¹⁴C)leucine for protein turnover measurements, as well as dramatic findings of Sender et al (1975) that diet itself could affect protein metabolism so greatly.

The scheme of work commenced with consideration of the (1-¹⁴C)leucine method itself. Although this method seemed to offer the most valid theoretical means of measuring human whole-body protein turnover, it was realised that the many techniques involved in making these measurements were potential sources of large errors. *Section 2* describes the technical problems associated with the method, and how these and other necessary correction factors were tested and resolved.

At the same time it was decided to measure the effect of a prolonged protein-free/low energy intake on protein turnover in obese patients, similar to the study of Sender et al (1975). It was felt that this was a sensible preliminary step, since significant errors were known to be associated with their (U-¹⁴C)tyrosine method. It was also planned to distinguish whether any change in protein turnover resulted from the low energy intake or from the prolonged absence of dietary protein, and this initial study is described in *Section 3*.

The findings in that study encouraged investigation of acute and diurnal changes in human protein metabolism. As exciting data from both normal and obese subjects emerged, the question of inherent circadian rhythms, diurnal changes in amino acid pools, and the patterns of the acute sensitivity of protein turnover to protein intake, were considered

SECTION 2 METHODS AND TECHNIQUES

2.1. CONSTANT INFUSION WITH L-(1-¹⁴C) LEUCINE

2.1.1. The Method

2.1.2. Subjects

2.1.3. Constant Infusion System

2.1.4. Measurement of specific radioactivity of
leucine in plasma

2.1.5. Measurement of specific radioactivity of
expired CO₂

2.1.6. Measurement of total CO₂ production (and
O₂ uptake)

2.1.7. Dietary intakes and protocols

2.1.8. A correction factor for incomplete excretion
of ¹⁴CO₂

2.2. (¹⁵N) GLYCINE

2.3. PRESENTATION OF RESULTS

2.1. CONSTANT INFUSION WITH L-(1-¹⁴C) LEUCINE : A PLASMA SPECIFIC ACTIVITY MEASUREMENT METHOD.

2.1.1. The Method

Consistent with the principles discussed in the Introduction, this method of measurement of whole body protein turnover requires particular skills and techniques, to obtain the following data:

- (i) A very constant and accurately known infusion rate of (1-¹⁴C)leucine (i DPM/hour).
- (ii) Plasma leucine specific activity at plateau (S DPM/ μ mole leucine).
- (iii) Expired CO₂ specific activity at plateau.
- (iv) Total CO₂ production rate throughout the measurement period.
- (v) A constant and accurately known intake of leucine (I).

As shown previously, the plasma leucine disposal rate can then be calculated from Equation 1, and this is used as a reasonable estimate of leucine flux (Q μ moles/hour) through the precursor pool.

$$i = QS \quad \text{or} \quad Q = i/S \quad \text{Equation 1.}$$

Leucine oxidation rate (E) is estimated by calculating the rate of excretion of ¹⁴CO₂ (e) from the product of CO₂ specific activity and total CO₂ production rate, and then assuming that the proportion of infused isotope being oxidised is a reflection of the proportion of leucine flux being oxidised, as in Equation 2.

$$e/i = E/Q \quad \text{Equation 2.}$$

Whole body protein synthesis (Z) and breakdown (B) can then be calculated from Equation 3. Flux, synthesis, breakdown, and oxidation rates, all initially measured and calculated in terms of leucine, can then be converted into 'whole body protein', a theoretical protein with an amino acid composition in weighted mean proportion to the composition of all body proteins. The conversion assumes that the leucine content of whole body protein is 8% (Block & Weiss, 1956).

$$Q = Z + E = B + I \quad \text{Equation 3.}$$

2.1.2. Subjects

Subjects were chosen on the following basis. Obese subjects were selected from patients attending Professor J.C. Waterlow's Obesity Clinic (University College Hospital, London), and control subjects from volunteer staff members (Dept. Human Nutrition, London School of Hygiene and Tropical Medicine). All subjects were of post child-bearing age, and had no disease known to be associated with protein metabolism. The infusion procedures were fully explained to the subjects, who signed consent forms confirming their prerogative to terminate the infusion at any time. The Isotope Advisory Panel of the Medical Research Council had approved the administration of up to 50 μCi of ($1\text{-}^{14}\text{C}$)leucine and subjects received amounts up to this maximum, usually by two infusions or a prolonged single infusion. Subjects were admitted as inpatients to the Hospital for Tropical Diseases, London NW1, either specifically for the ($1\text{-}^{14}\text{C}$)leucine infusion, or for a period (usually 6 weeks) of inpatient weight reduction. These studies were approved by the Ethical Committee of University College Hospital, London.

2.1.3. Constant Infusion System

The isotope infusion solution was prepared by mixing the required total infusion dose (eg. 25 μCi) of L-(1- ^{14}C)leucine (Radiochemical Centre, Amersham, Bucks, U.K.) with approximately 25 ml of sterile 0.9% saline, and injecting this solution through a millipore filter (0.22 μm) into a sterile 25 ml disposable syringe, and was thus ready for use.

The constancy of isotope infusion is crucial for this technique, and even small changes in infusion rate, lasting only a few minutes, were able to alter plasma specific activity, and hence spoil plateau measurements. Initially the isotope/saline solution was infused directly intravenously into subjects through fine bore Silastic tubing, using a constant infusion syringe pump. This system proved unsatisfactory, as its delicacy restricted the subjects movements during the 8 - 10 hours of infusion, and the combination of changes in hydrostatic pressure in the tubing (as a result of subject movement relative to the syringe pump), and the compliance of the tubing itself, produced an unacceptable variability in the rate of isotope delivery at needle tip.

The constant infusion system that was eventually developed involved a constant rate intravenous infusion of N-saline controlled by a Tekmar T92H Volumetric Infusion Pump (Tekmar Medical Ltd, Milton, Oxon, U.K.), usually run at a rate of 50 ml/hour. Through a side arm of the intravenous line, a syringe pump infused the (1- ^{14}C)leucine/saline solution via a long 25 gauge needle. The great advantage of this system, proved to be the reliability and consistency of the Tekmar infusion pump. There was never more than $\pm 0.2\%$ variation from a measured mean delivery rate (C.V. = 0.13%), and the built-in alarm systems for air bubbles, blockages, completion of infusion solutions, and power failure actually rescued some infusions.

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The rate of constant infusion of (1-¹⁴C)leucine was measured for each infusion by immersing the infusate syringe needle tip directly into a series of scintillation vials each containing 10 mls of scintillant (Triton X/toluene 1:2 v/v with 0.5% 2,5 diphenyloxazole) for an exactly determined time interval, and the rate in DPM/hour determined.

2.1.4. Measurement of specific radioactivity of leucine in plasma

Blood samples were usually taken from a forearm vein, via an indwelling intravenous cannula thus facilitating repeated sampling, even with the subject remaining asleep. Once it was anticipated that plasma leucine specific activity had achieved plateau (usually about 6 hours after commencement of infusion), 10 ml blood samples were taken hourly or even more frequently, transferred to heparinised tubes, immediately plunged into ice, and as rapidly as possible spun in a refrigerated centrifuge, the plasma being retained and stored frozen.

The plasma samples were prepared in the following manner, for measurement of leucine specific radioactivity. The plasma proteins from a known volume of plasma (3.0 ml), were precipitated with 1 ml of 10% perchloric acid, then spun and separated off, and the pH of the retained supernatant readjusted to approximately pH 4 with 10% potassium hydroxide. After allowing this to stand cold for an hour, it was centrifuged, the supernatant freeze dried, and subsequently reconstituted to 0.9 ml with pH 2.2 citrate buffer. The sample was then loaded on to an automated amino acid analyser (Locarte Ltd, London) and an accurately measured proportion of the column effluent diverted by a stream splitter and fraction collector. The fractions containing the leucine peak were transferred to glass scintillation vials, a

10 ml aliquot of 'Tritosol' scintillant (0.3% 2,5-diphenyloxazole in a mixture of ethanediol/ethanol/Triton X 100/xylene in v/v proportions of 1:2.9:6.9:10.8) was added, and the vials counted for radioactivity in one of three available liquid scintillation counters (Beckman LS 150, Packard 2420, and a Nuclear Chicago Delta 300). In this way the plasma leucine specific activity was measured, for each sample. Furthermore, by adding 0.4 μ moles of cycloleucine to the initial 3.0 ml of plasma and measuring its proportional recovery from the amino acid analyser, plasma leucine concentration (and hence pool size) was estimated for some subjects.

2.1.5. Measurement of specific radioactivity of expired CO_2

The rate of leucine oxidation can be estimated, as outlined in the Introduction, by measuring the rate of expiration of $^{14}\text{CO}_2$. This was achieved by measuring the proportion of $^{14}\text{CO}_2$ in expired CO_2 (ie. its specific activity), and the rate of total CO_2 production.

The specific radioactivity of expired CO_2 reaches a plateau value soon after plasma leucine reaches its plateau specific activity (Fig. 2.1). Subjects filled a 2 litre polythene bag with expired breath, and this was then bubbled into 3.0 mls of 1M hyamine hydroxide/ethanol solution (1:2, v/v) with 0.1% phenolphthalein as indicator (Kaihara & Wagner, 1968) in a glass scintillation vial, until the solution became colourless, indicating saturation of the hyamine with breath CO_2 . For each leucine infusion, a new batch of scintillation vials, each containing 3.0 mls of freshly mixed solution was prepared, and several vials were titrated against 0.15N HCl to determine the mean molar combining affinity for CO_2 per vial.

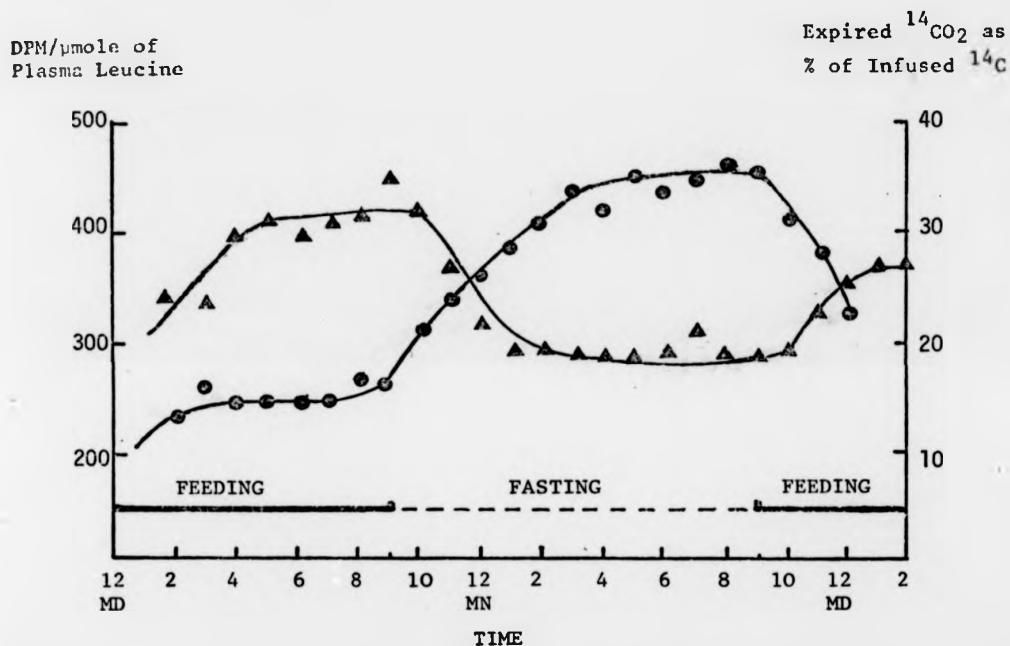


FIGURE 2.1. The rise to plateau of plasma (1-¹⁴C)leucine specific activity (●), and percent of infused (1-¹⁴C)leucine appearing as ¹⁴CO₂ (▲). The changes in the plateaux result from feeding and fasting. (Patient F.B.)

An aliquot (10 ml) of scintillant (0.4% 2,5-diphenyloxazole in toluene) was added to each CO₂-saturated vial, and the radioactivity measured by counting in one of the three available liquid scintillation counters. Appropriate quenching correction factors were applied, quench curves for each machine being periodically checked using external standards. Thus each vial contained a known molar quantity of

CO_2 and its specific activity (DPM/mole CO_2) was calculated. Once at plateau the respiratory CO_2 specific activity was measured at half hourly intervals.

2.1.6. Measurement of total CO_2 production (and O_2 uptake)

The rate of CO_2 production was initially measured using a mouthpiece and valve connected to an 80 litre polythene bag and in this way the subject's expired air was collected over timed intervals on several occasions during the infusion period. The collection bag was expelled into a Morgan Mobile BMR System (containing an Analytical Development Co. 854 dual range infra-red CO_2 analyser, a Servomex OA272 oxygen analyser, and a Parkinson Cowan CD4 gas volume meter fitted with photo pulse generator) and gas volume, percent CO_2 and percent O_2 were determined. A variant of this technique involved the subject breathing directly through a Max Planck respirometer to determine total expiratory rate, and simultaneously collecting several small (2 litre bags) samples of expired air for CO_2 and O_2 analysis.

This whole approach proved most unsatisfactory. The awareness and discomfort of mouthpiece and valve were shown to cause marked overbreathing and high CO_2 output values, distinctly unrepresentative of the subject's steady state CO_2 output (see Fig. 2.2). In addition the polythene bags were found to be significantly permeable to CO_2 (Fig. 2.3).

These problems were overcome by developing a ventilated tent system that allowed the patient to sit, lie, relax, talk, eat, watch T.V., or even sleep, and yet simultaneously have continuous measurement of CO_2 output and O_2 uptake rates. This system is shown in operation, in the accompanying photographs.

FIGURE 2.2. CO_2 output, as influenced by the respiratory gas collection systems, measured in the same patient (M.B. ♀ age 43). The ventilated tent value (10.17 l/hr) is designated 100 %.

- A ventilated tent (10.17 l/hour)
- B ventilated hood (10.70 l/hour)
- C valve & Douglas bag (13.78 l/hour)

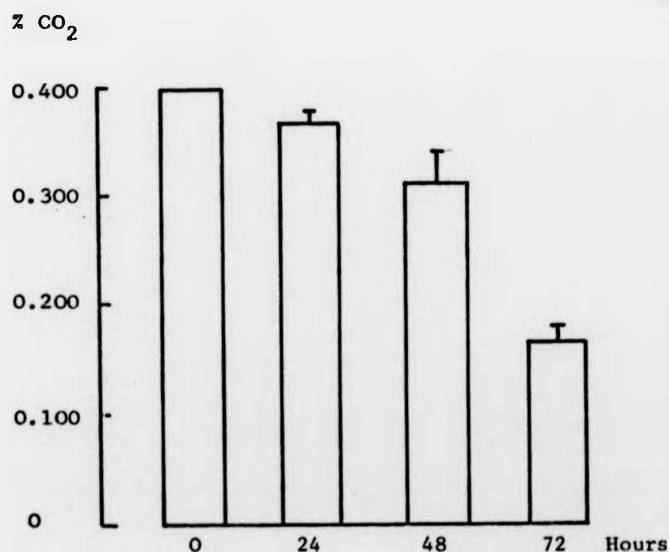
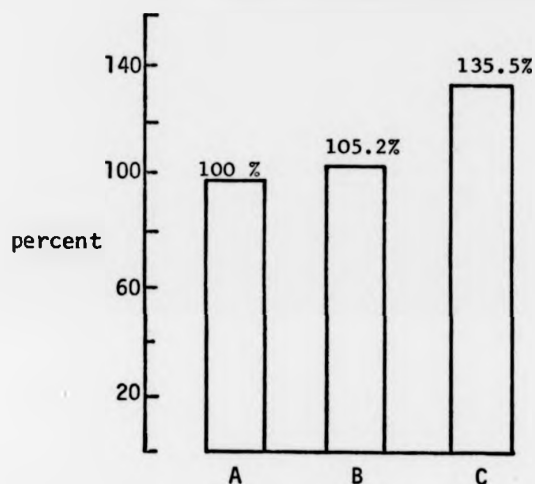


FIGURE 2.3. Mean (\pm S.D.) percentage concentration of CO_2 in 5 polythene gas collection bags (2 litre), initially filled with standard 0.400% CO_2 .

The great advantages of this system were only fully appreciated when investigation of diurnal patterns of protein turnover and energy metabolism were commenced (see Results 2). A major limitation in past studies where estimations of protein oxidation or energy expenditure have been attempted, has often been the inability to obtain accurate measurements or collect samples representative of 12 hours or 24 hours CO_2 output and O_2 uptake. Our leucine infusions were prolonged up to 40 hours, and yet CO_2 output and O_2 uptake were able to be monitored almost continuously throughout. Furthermore it was the development of this facility that made it possible to investigate compartmentation of energy metabolism on a diurnal basis, as described in Part B of this thesis.

The system (see Fig. 2.4) consisted of an oxygen tent set up around the subject's bed, and connected by corrugated piping, to a mixing cone, the CD4 gas volume meter, a 2-way valve, and finally to a high pressure 4MS-8 centrifugal blower motor (Air Control Installations Ltd, Chard, U.K.). A sampling pump withdrew air from the mixing cone, circulated it through the CO_2 and O_2 analysers, and returned it to the inlet of the CD4 gas volume meter. As well as a digital readout facility for rate of gas flow, CO_2 concentration and O_2 concentration, these outputs were also fed into a multichannel pen recorder, for continuous monitoring of these parameters.

The high pressure 4MS-8 electric blower motor was chosen because it sucked air at a constant rate of some 4000 litres/minute. A 2-way valve on the input side of the motor acted as a controlled Y-junction so that the required flow rate through the system (usually set at about 70.0 l/minute) came through one arm of the valve, and room air (ie. at some 3,930 l/minute) came through the other arm. By operating the 2-way valve in this manner so that the required flow rate through the

The (1-¹⁴C)leucine infusion system and ventilated tent system in operation.



A. Daytime



B. Night-time

('Control' colleague Prof. R.W.S.)

The (1-¹⁴C)leucine infusion system and ventilated tent system in operation.



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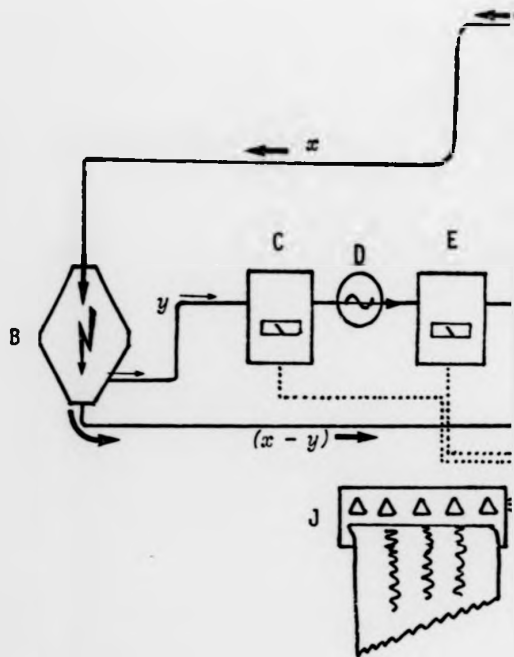
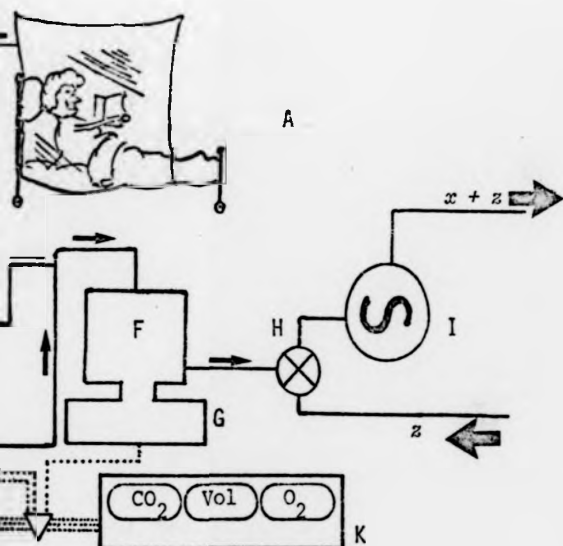


FIGURE 2.4. The Ventilated Tent System



- | | | | |
|---|--|-----|--------------------------|
| A | Patient in ventilated tent | x | Main line flow from tent |
| B | Mixing/Sampling cone | y | Sampling line |
| C | Infra-red CO ₂ Analyser | z | Room air intake |
| D | Sampling line pump | x+z | Total out-flow |
| E | Paramagnetic O ₂ Analyser | | |
| F | CD4 Gas Volume meter | | |
| G | Photo pulse generator | | |
| H | Flow-rate control valve | | |
| I | High Pressure 4MS-8 Centrifugal blower motor | | |
| J | Multichannel pen recorder | | |
| K | CO ₂ /Volume/O ₂ Digital display | | |

system was only a very small proportion (approx. 1.75%) of total flow through the valve and blower motor, extremely constant flow rates were achieved.

It became apparent that constancy of flow was crucial to precision of results, as total CO_2 output is calculated as a product of the system's ventilation rate and the small percentage changes in CO_2 concentration. Earlier use of rheostat controlled electric motors in line with the system sucking 70 litres/min directly through both system and motor, resulted in substantial flow variability (eg. 70 ± 1.2 litres/minute) due to mains voltage fluctuations and minor resistance changes in the system itself. Use of the high pressure motor and 2-way valve as described above, dramatically improved this (eg. 70.0 ± 0.2 litres/minute) with a coefficient of variance of only 0.16%.

The CD4 gas volume meter with photo pulse generator allowed accurate monitoring and continuous recording of this constant flow rate. It had been found that a flow rate of about 70 litres per minute adequately ventilated the tent for comfort, yet still permitted sufficiently large changes in O_2 and CO_2 concentrations in the room air as it passed through the tent, for accurate measurement. The output signals from both CO_2 analyser and O_2 analyser were amplified so that a full scale pen deflection on the multichannel pen recorder represented only a 1.00% change in absolute concentrations of O_2 and CO_2 from room air. This permitted excellent precision and enabled the continuously monitored O_2 and CO_2 percentages in the ventilated air to be measured within $\pm .005\%$ (absolute) of a mean.

After trying various hoods and helmets, it was found that an oxygen tent with a volume of approximately 1000 litres produced the best results, being large enough for both complete patient comfort and

damping of respiratory fluctuations in O_2 and CO_2 concentrations, yet small enough to produce accurately measurable changes in O_2 and CO_2 levels when the tent was ventilated at about 70 litres/minute.

Immediately prior to each infusion, and every 6 hours during an infusion, the CO_2 analyser was calibrated against a 0.50% CO_2 /99.50% N_2 gas mixture (British Oxygen Company - Special Gases. Hy-line Grade analysed and certified $\pm 0.02\%$ absolute).

The accuracy of the entire ventilated tent system was tested on a number of occasions, by burning ethanol at an accurately measured constant rate in the tent, and comparing the theoretically predicted values of O_2 consumption and CO_2 production with those measured and recorded. The rate of C_2H_5OH combustion (measured by a Sartorius 1212MP electronic balance) was set so that O_2 consumption and CO_2 production rates were similar to those in adults. The results below demonstrate the rather impressive accuracy (eg. only a relative difference of 0.31% between measured mean CO_2 production rate and its predicted value) and excellent precision (eg. C.V. of only 0.22% for CO_2 production measurement and 1.45% for O_2 consumption measurement) achieved by the whole system. (see predicted and measured rates on following page).

The development and details of this system are described here in detail, because it was really this ability to make prolonged continuous measurements of rates of O_2 uptake and CO_2 output over many hours, that enabled confident measurements to be made of diurnal changes in protein oxidation rates and hence protein synthesis rates, and that subsequently led to investigation of hourly and diurnal changes in deposition and mobilisation of fuels in energy metabolism.

PREDICTED RATES

<u>MEAN RATE C₂H₅OH COMBUSTION</u>	<u>TEMP/PRESSURE</u>	<u>THEORETICALLY PREDICTED VALUES</u>	
		CO ₂ OUTPUT RATE litres CO ₂ /hr	O ₂ UPTAKE RATE litres O ₂ /hr
g/hour	°C/mmHg		
9.592	22°C/773mmHg	9.831	14.860

MEASURED RATES

	<u>CO₂ PRODUCTION RATE</u> l/hour	<u>O₂ UPTAKE RATE</u> l/hour
at 30 mins	9.818	14.904
at 45 mins	9.777	14.477
at 1 hour	9.806	14.679
Mean ± S.D.	9.800 ± 0.021	14.687 ± 0.214
C.V.	0.22 %	1.45 %
$\frac{\text{Measured Rate}}{\text{Predicted Rate}} \times 100 \%$	99.69 %	98.84 %

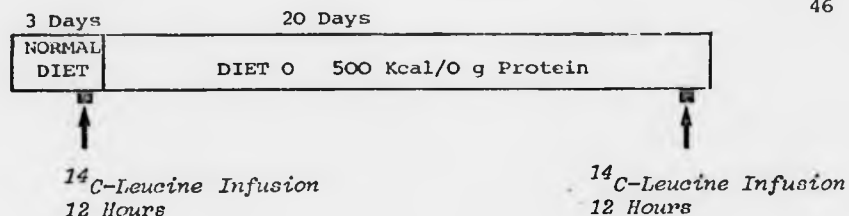
2.1.7. Dietary intakes and protocols

For all infusions it was necessary to know accurately the intakes of leucine, protein, and energy and to ensure that they were administered at a constant rate over a daily feeding period. The rate of leucine intake had to be known for calculation of whole body protein breakdown rates, using Equation 3. It was important to know protein and energy intakes because of their influence on protein turnover - one of the effects investigated in this thesis. Different dietary regimes were therefore devised to supply planned quantities of protein and energy. The details of diets are summarised below, but they were usually either aimed at maintenance of weight (the 'Normal' Diets) or weight reduction in which case they contained 500 Kcal (2.1 MJ) and varying quantities of protein, depending on the experimental protocol (eg. Diet P, Diet O, etc).

Most of the subjects can be classified into one of several different experimental groups, distinguished by dietary regime and/or method of protein turnover measurement. Their experimental protocols are shown below, followed by details of the specific diets used.

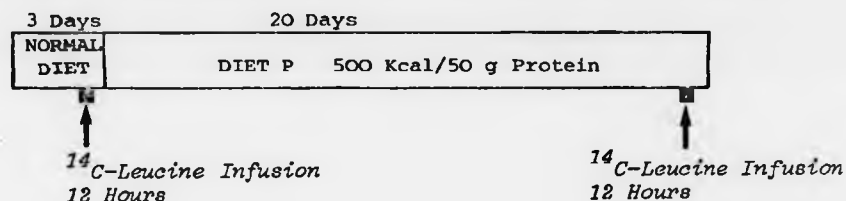
1. 'DIET O' group of obese patients (see Section 3)

The obese patients of this group were initially given a weight maintenance diet 'Normal' Diet A or B (detailed below) over the first 3 days. Protein turnover was then measured by (1-¹⁴C) leucine infusion on the 3rd day, the infusion lasting 12 hours and co-inciding with the feeding 12 hour period in which 'Normal' Diet A or B was administered in 12 equal hourly portions. Then followed a 3 week period on Diet O, aimed at supplying 500 Kcal (2.1 MJ) and 0 g protein per day. On the 20th day of this diet, a second infusion of (1-¹⁴C) leucine remeasured protein turnover rate, again over the feeding 12-hour period of Diet O.



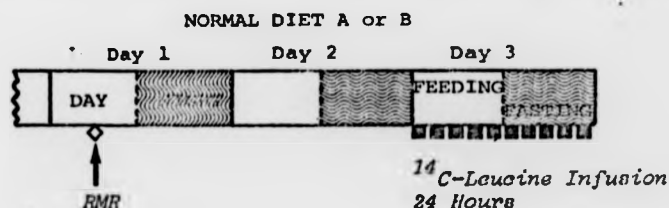
2. 'DIET P' group of obese patients (see Section 3)

These obese inpatients adhered to an identical protocol to the Diet O group except that their 3 weeks weight reducing diet was Diet P, aimed at supplying 500 Kcal (2.1 MJ) and 50 g protein per day.



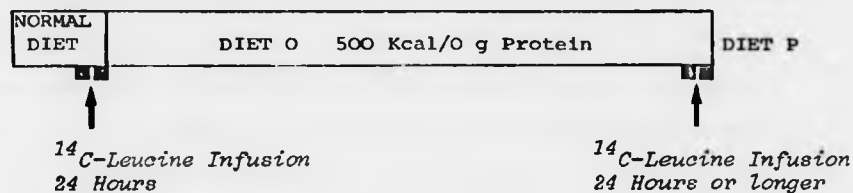
3. 'DIURNAL' group of obese patients (see Section 4)

These obese patients were usually admitted for 2 or 3 days only. On the first day resting metabolic rate was measured, and their daily energy intake on 'Normal' Diet A or B tailored to meet estimated energy requirement. On day 3, protein turnover was measured by ($1\text{-}^{14}\text{C}$) leucine infusion prolonged over 24 hours, including the 12-hour feeding period in which 'Normal' Diet was administered in 12 equal hourly portions, followed by 12 hours of fasting (ie. 'post-absorptive state').



4. 'DIET O - DIURNAL' group of obese patients

The protocol for this group was identical to that for Diet O group, except that both infusions with (1-¹⁴C) leucine were prolonged over at least 24 hours, and for some patients a protein refeeding regime with Diet P was commenced after 20 days on Diet O.

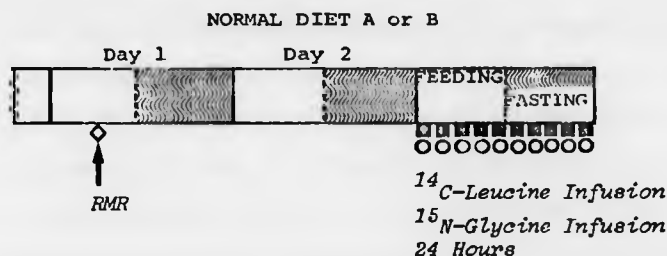


5. ¹⁵N-GLYCINE group of obese patients (see Section 5)

Most of these obese patients initially had 3 days on 'Normal' Diet A or B and then a period of 3 weeks or more on one of several 500 Kcal (2.1 MJ) per day diets, including Diet O, Diet P, 500 Kcal/25 g protein, 500 Kcal/25 g gelatin. The distinguishing common feature was that protein turnover was repeatedly measured at frequent intervals using the (¹⁵N)glycine method. In some patients protein turnover was measured simultaneously with (1-¹⁴C) leucine and (¹⁵N)glycine.

6. 'CONTROL' group (see Section 4)

These subjects were non-obese departmental colleagues. The energy intake for their 'Normal' Diet was estimated by measurement of each subject's resting metabolic rate one or two days prior to the infusion. Protein turnover was measured by simultaneous continuous 24 hour infusion of both ($1-^{14}\text{C}$) leucine and (^{15}N) glycine, thereby giving values for both feeding 12-hour phase, and the fasting (post-absorptive) 12-hour period, from two different measurement methods. In one subject (R.S) protein turnover measurement was commenced with the night (fasting) phase first.



7. ' $(1-^{13}\text{C}, ^{15}\text{N})$ LEUCINE' group (see Section 7)

This was also a group of departmental colleagues, participating in a joint study (Rennie *et al*, 1980). There were 2 series of ($1-^{13}\text{C}, ^{15}\text{N}$) leucine infusions, the first with each subject on a 'Normal' Diet, with the infusion measuring protein turnover during the feeding phase. For the second series the subjects were in a fasting state, having fasted overnight and continued their fast through the day time measurement period. Muscle biopsies were taken on 2 occasions from both thighs during each infusion.

Most patients began their dietary protocol with a period on 'Normal' Diet. This diet was intended to supply sufficient energy for weight maintenance, as well as an adequate or excess intake of protein, usually about 70g protein per day. For the earlier infusions, such as the first (1-¹⁴C)leucine infusion for each of the obese patients of Diet P group and Diet O group, the energy intake during the 3 days of 'Normal' Diet was arbitrarily set at 2000 Kcal (8.4 MJ)/day for women, and 2500 Kcal (10.5 MJ)/day for men. Later this protocol was changed because of the presumed difference in energy requirements for patients with different degrees of obesity, the use of non-obese 'control' patients, and particularly because of increasing interest in energy expenditure measurements. The practice then adopted was to make a preliminary measurement of the resting rate of energy expenditure (from O₂ consumption measurements) for each patient, on the first day of their 'Normal' diet, and adjust the energy content of the diet to equate with this. The individual values of the 'Normal' Diet energy intakes for each patient are given in the Results, but usually amounted to a daily energy intake of approximately 1,600 Kcal (6.7 MJ).

The 'Normal' Diet was either a liquid milk-based diet ('Normal' Diet A) formulated from 'Slender' (a commercial powdered-milk based food replacement product - Carnation Foods Ltd, London, U.K.), milk, double cream, and fruit juice, or alternatively a sandwich diet ('Normal' Diet B) composed of bread or crackers, butter, cheddar cheese, and tomato. Most patients used 'Normal' Diet A as it was only in later stages that 'Normal' Diet B was developed to cope with the occasional patient unable to tolerate the large quantities of milk in 'Normal' Diet A. Although the component quantities were varied to supply estimated energy requirements for each patient, the following

table gives a typical nutrient composition for the 'Normal' Diets A and B.

TABLE 2.1. Typical daily nutrient intake on 'Normal' Diets A and B.

	ENERGY Kcals (MJ)	PROTEIN g	LEUCINE mmoles	N g	CHO g	FAT g
'NORMAL' DIET A	1,932 (8.08)	74.8	55.18	11.73	250.4	75.9
'NORMAL' DIET B	1,902 (7.96)	74.3	53.45	12.15	137.2	121.6

The weight-reducing diets all contained 500 Kcal (2.1 MJ) per day, and were designed to vary only in protein content, which was nevertheless accurately known. Diet P, aimed at supplying 500 Kcal (2.1 MJ) and 50 g protein per day, was formulated entirely from skimmed milk and 'Slender'. Diet O was composed of a glucose syrup 'Hycal' (Beecham Products, Middlesex, U.K.) and supplied 500 Kcal (2.1 MJ) and no protein each day. Occasionally other diets were supplied, particularly for a group of obese patients having repeated (^{15}N)glycine measurements of protein turnover, and although their daily energy intake remained at 500 Kcal (2.1 MJ) their protein intake was changed to 25 g protein, and even 25 g gelatin. These diets are detailed further in the relevant Results sections. The following table (Table 2.2) shows the composition of Diet P and Diet O.

TABLE 2.2. Typical daily nutrient intake with the two main weight reducing diets, Diet P and Diet O.

	ENERGY Kcal (MJ)	PROTEIN g	LEUCINE mmol	N g	CHO g	FAT g
DIET P	541.8 (2.27)	49.6	37.98	7.77	85.1	1.7
DIET O	504.8 (2.1)	0	0	0	132.0	0

Whilst the energy, protein, carbohydrate, and fat composition of the diets were calculated from food tables (McCance and Widdowson's *The Composition of Foods*, Vol 3 and Vol 4), duplicate samples were taken from all diets for all subjects, homogenised and analysed for N by the Kjeldahl method, for fat by Soxhlet extraction method, and for energy by bomb calorimetry. Dietary leucine was estimated by hydrolysis of an aliquote from the homogenate in 6N HCl at 110°C for 48-72 hours followed by ion exchange chromatography in an automated amino acid analyser using 0.1 µmol norleucine as standard. These analyses confirmed the food table values used.

A significant correction, accounting for digestibility, still had to be applied to these intake values whether derived from food tables or direct analysis. This was because the intake values (I) for leucine or protein, used for calculation of whole body protein breakdown rates, needed to be the actual quantities entering the 'metabolic pool'. The same correction was also required for calculation of energy metabolism values. Appropriate digestibility factors (Watt & Merrill, 1963; Southgate & Durnin, 1970) for the protein, fat, and carbohydrate content in each of the constituent foods in every subject's diet were applied.

Thus the intake values of leucine, protein, nitrogen, carbohydrate, and fat presented in the Results sections and used for calculation of protein breakdown rates, and fuel and energy balances, represent as closely as possible the quantities immediately available for metabolism.

2.1.8. A correction factor for incomplete excretion of $^{14}\text{CO}_2$

One of the disadvantages found in using (U- ^{14}C)tyrosine as an amino acid tracer, was that some of the labelled carbon released during tyrosine oxidation was not immediately excreted as $^{14}\text{CO}_2$, but was probably recycled via fatty acid synthesis and gluconeogenesis before being eventually excreted. When (1- ^{14}C)leucine is oxidised, the ^{14}C is irreversibly removed (as $^{14}\text{CO}_2$) in the second step of the oxidative pathway, and the $^{14}\text{CO}_2$ excreted with expired CO_2 . Nevertheless it has been found that the $^{14}\text{CO}_2$ produced by (1- ^{14}C)leucine oxidation is not completely excreted, a small proportion being retained probably in the bone bicarbonate pool and other pathways. Estimates of this proportion range from 13 - 30% (Clague *et al*, 1979; James *et al*, 1976; Winchell *et al*, 1970; Issekutz *et al*, 1968).

Accurate determination of the proportion of leucine flux contributed by leucine oxidation, requires an estimate of total rate of $^{14}\text{CO}_2$ production at the actual site of oxidation. A correction factor to account for the small proportion of $^{14}\text{CO}_2$ not excreted with expired CO_2 needs to be applied to the measured rate of $^{14}\text{CO}_2$ output. The value of this factor can be obtained from the proportional recovery of $^{14}\text{CO}_2$ during an infusion of ^{14}C -labelled bicarbonate. James *et al* (1976) showed an 80% recovery of infused $^{14}\text{CO}_3^{2-}$ during short (10 hour) infusion periods in 3 subjects. Very recently Clague *et al* (1979) have obtained 86.5% recovery in 5 patients using primed dose 2-hour infusions.

When our investigations were commenced into the possibility of diurnal changes in protein turnover, and infusions were extended over 24 hours and even up to 40 hours to cover both day and night and periods of feeding, fasting, and refeeding, it was felt that the correction factor for $^{14}\text{CO}_2$ recovery should be measured over similarly extended periods in case diurnal metabolic changes affected its value.

Ten ^{14}C -labelled bicarbonate infusions were carried out, most lasting some 36 hours to cover 3 twelve hour periods - ie. feeding, fasting, and refeeding. Both obese and 'control' subjects were represented and indeed five of the subjects also belonged to groups of patients who had been infused with (1- ^{14}C)leucine for measurement of their protein turnover rates. To exclude any dietary effect on recovery of $^{14}\text{CO}_2$, subjects were put on one of three dietary regimes, identical to the regimes used for the protein turnover groups - ie. 'Normal' Diet (mean = 1675 Kcal/75 g Protein), Diet P (500 Kcal/50 g Protein) and Diet O (500 Kcal/0 g Protein). As with the diets for (1- ^{14}C)leucine infusions, these intakes were taken in 12 equal hourly portions over a 12 hour period, followed by 12 hours of fasting, after which time the same diet was recommenced.

For one of the subjects (R.S) the ^{14}C -labelled bicarbonate infusion was commenced at night and so the fasting 12 hour period was the first period for measuring the proportion of $^{14}\text{CO}_2$ recovered.

The salient points of the infusion technique are briefly summarised here. A total dose of approximately 10 - 20 μCi of ^{14}C -labelled sodium bicarbonate (Radiochemical Centre, Amersham, Bucks, U.K.) was passed by a 1 ml syringe and needle into a 20 ml syringe filled with N-saline which already contained approximately 5 mmol of sodium bicarbonate. This solution was sterilised using a 0.22 μm millipore filter and infused using a syringe pump and Tekmar T92H Volumetric Infusion Pump, as

described for the (1- 14 C)leucine infusions. The rate of CO_2 output was measured by the ventilated tent system described earlier, and CO_2 specific radioactivity was estimated by liquid scintillation counting. Thus the rate of $^{14}\text{CO}_2$ output could be expressed as a percentage of rate of ^{14}C -labelled bicarbonate infusion.

The percentage recovery of infused ^{14}C -bicarbonate is shown in the results below. In each subject measurements were usually made hourly throughout the entire infusion, but the results below show the mean values for each 12 hour period for each subject

TABLE 2.3. Mean percent recovery of infused $\text{H}^{14}\text{CO}_3^-$ during feeding, fasting and refeeding 12-hour periods, for subjects on different dietary regimens

SUBJECT	O=Obese C=Control SEX F/M	DIETARY REGIMEN Kcal/Protein per 24 hours	MEAN % RECOVERY OF INFUSED H^{14}CO_2		
			Feeding 12 Hours	Fasting 12 Hours	Refeeding 12 Hours
<u>A. 'NORMAL' DIET GROUP</u>					
R.P.	O F	1300/71	87.1 %	91.2 %	92.8 %
M.B.	O F	1400/72	90.7 %	97.1 %	-
R.S.	C M	2500/80	90.2 %	93.8 %	-
M.L.	O F	1550/77	95.7 %	103.7 %	-
M.M.	O M	1600/79	80.4 %	90.7 %	93.2 %
G.C.	O F	1700/70	-	84.2 %	88.7 %
<u>MEAN \pm S.D.</u>			88.8 % \pm 5.6	93.5 % \pm 6.5	91.6 % \pm 2.5
<u>B. DIET P GROUP</u>					
A.G.	O F	500/50	87.0 %	88.2 %	86.7 %
<u>C. DIET O GROUP</u>					
M.L.	O F	500/O	90.1 %	99.4 %	94.2 %
M.W.	O F	500/O	81.2 %	92.1 %	-
E.S.	O F	500/O	91.0 %	85.8 %	92.4 %
<u>MEAN \pm S.D.</u>			87.4 % \pm 5.4	92.4 % \pm 6.8	93.3 % \pm 1.3

For each of the 3 feeding/fasting phases, there was no statistically significant difference between the mean values of the 3 dietary groups.

Combining the values for all 10 subjects for each 12 hour phase (Table 2.4) indicates a constant proportional recovery over the whole 36 hour period.

TABLE 2.4. Mean percent recovery of Infused $\text{H}^{14}\text{CO}_3^-$ for all 10 Subjects

	FEEDING 12 hrs	FASTING 12 hrs	REFEEDING 12 hrs
MEAN % RECOVERY	88.2 %	92.6 %	91.3 %
± S.D.	± 4.9	± 6.1	± 2.9

Paired t-testing showed no significant difference between these mean recovery rates apart from the comparison between feeding and fasting periods which showed $0.01 < p < 0.05$. It seems unlikely that this is a genuine difference, particularly since the refeeding period value of 91.3% is not significantly different from either of the other periods. The mean value of the percentage recovery of infused $\text{H}^{14}\text{CO}_3^-$, calculated from all subjects in all phases, is 90.7% (standard deviation ± 5.3), indicating a mean loss of 9.3% of infused ^{14}C , into bone or other metabolic pathways.

It can be concluded that when $(1-^{14}\text{C})$ leucine (or $(1-^{13}\text{C})$ leucine) is infused for measurement of protein turnover, 90% of $^{14}\text{CO}_2$ (or $^{13}\text{CO}_2$) produced from decarboxylation of the leucine keto acid (2-keto isocaproate) is excreted and can be measured, 10% being retained in the body, probably mixing with the bicarbonate pools of bone or other pathways.

Consequently, a correction factor of 100/90 has been applied to all the measured rates of $^{14}\text{CO}_2$ output in the results in this thesis,

as it is an estimate of $^{14}\text{CO}_2$ production rate in the metabolic pool that is required for calculation of valid leucine oxidation rates.

2.2. (^{15}N)GLYCINE - AN EXCRETED END PRODUCT METHOD

Although development and use of the ($1\text{-}^{14}\text{C}$)leucine constant infusion method has been the mainstream of protein turnover investigation in this thesis, some measurements were also made using (^{15}N)glycine, particularly for comparative purposes. It initially became clear, using ($1\text{-}^{14}\text{C}$)leucine constant infusions, that the level of dietary protein intake could significantly affect whole body protein synthesis, at least over a number of weeks. Consequently it was decided to measure protein turnover using (^{15}N)glycine, as this not only offered an alternative and independent (ie. an excreted end-product method) estimate of protein turnover which could confirm the changes shown with the ($1\text{-}^{14}\text{C}$)leucine method, but being a stable isotope it had the advantage of being able to be administered on numerous occasions (eg. every two days) and could therefore be used to monitor just how rapidly any dietary induced changes in protein turnover occurred.

Principles involved in the use of a urinary excreted end-product method, the choice of end product, and the choice of isotope administration technique (ie. single dose, multiple dose, constant infusion, etc), have been mentioned earlier and are discussed in detail by Waterlow, Garlick and Millward (1978) in their book *Protein Turnover in Mammalian Tissues and in the Whole Body*. The practical aspects of how (^{15}N)glycine was administered and the abundance of ^{15}N in a urinary end product measured for these patients, require a brief outline.

In a number of both obese and control subjects, simultaneous measurements of protein turnover were made by both ($1\text{-}^{14}\text{C}$)leucine constant infusion, and (^{15}N)glycine, where the dose of (^{15}N)glycine was administered in a number of different ways. These studies, using two simultaneously administered independent methods, not only confirmed the

changes in whole body protein turnover induced by different levels of dietary protein over a number of weeks, but allowed a comparison of different administration techniques for (^{15}N)glycine. The following techniques were used, each case having a ($1\text{-}^{14}\text{C}$)leucine infusion at the same time:

- continuous I.V. infusion (^{15}N)glycine;
- multiple oral dose (^{15}N)glycine;
- single I.V. dose (^{15}N)glycine;
- single oral dose (^{15}N)glycine.

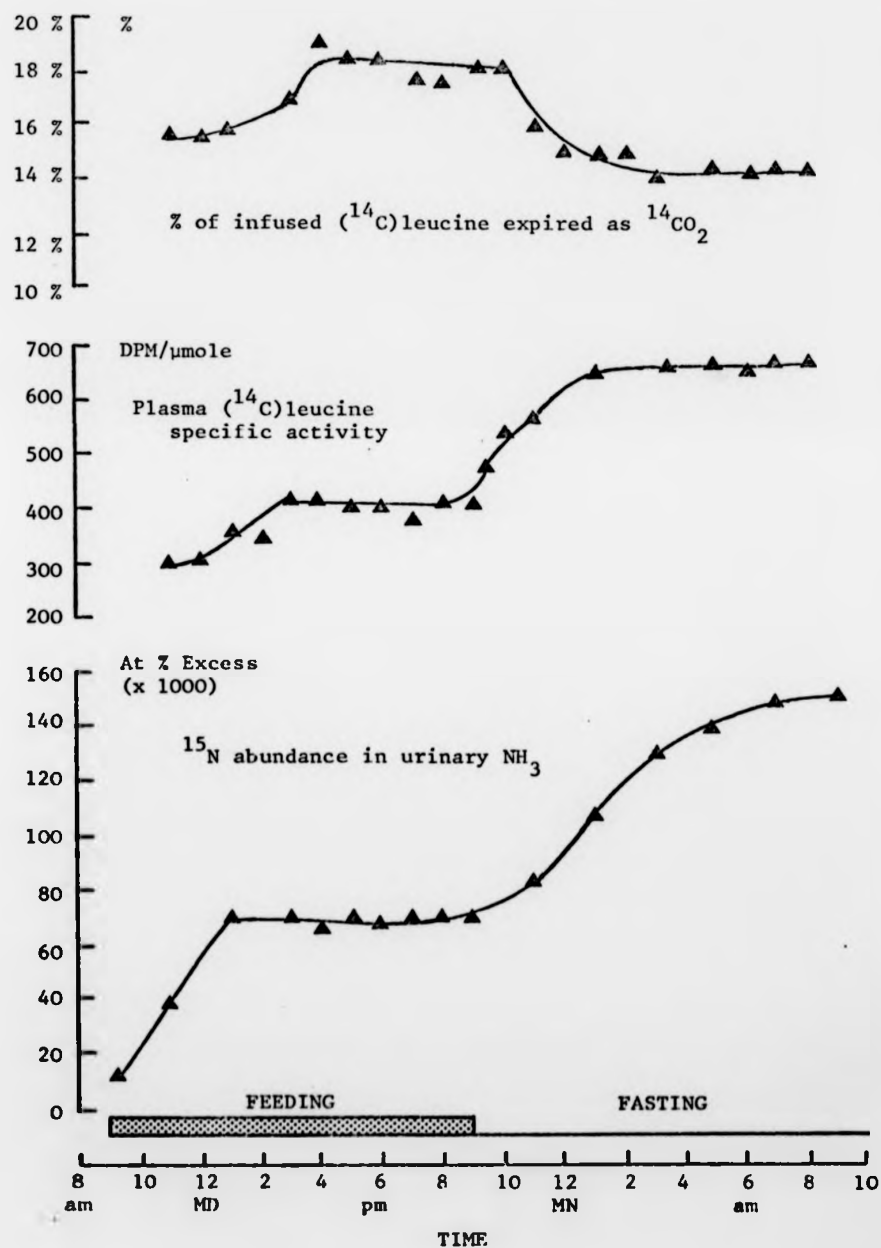
When (^{15}N)glycine was used to measure protein turnover on numerous and repeated occasions in the same subject (without a simultaneous ($1\text{-}^{14}\text{C}$)leucine infusion), the dose was usually administered by oral or intravenous single dose technique.

Continuous intravenous infusion of (^{15}N)glycine

An accurately weighed quantity of (^{15}N)glycine (usually about 300.0 mg of (^{15}N)glycine, 95.2 atoms % excess, from Prochem Division of British Oxygen Co, London, U.K.) was dissolved in an accurately measured volume of an N-saline solution, which already contained the required total dose of ($1\text{-}^{14}\text{C}$)leucine dissolved in 2.5 ml of an amino acid mixture. This infusate was sterilised using a 0.22 μm millipore filter, and infused by syringe pump and infusion system, as described for ($1\text{-}^{14}\text{C}$)leucine infusions. The accurately known infusion rate was approximately 1.0 mg ^{15}N (and 1.5 μCi ($1\text{-}^{14}\text{C}$)leucine) per hour for most subjects, and for this group of subjects all infusions were of 24 to 30 hours duration, covering a 12 hour feeding phase and a 12 hour fasting phase.

A 'pretest' urine sample was taken immediately before the commencement of the infusion, and during the infusion urine was collected at

FIGURE 2.5. Control subject (J.C.W.): Protein turnover measured over 24 hours, with both (1- 14 C)leucine I.V. infusion method (15N)glycine I.V. infusion method



accurately time intervals, usually hourly during waking hours, and every two hours during the sleeping phase. The abundance of ^{15}N in urinary ammonia was measured (detailed below), its rise to plateau noted (see Fig 2.5) and then subsequent values used to calculate a plateau mean (S) for the ^{15}N abundance. The flux (Q) could then be estimated, in an exactly analogous calculation to that used for (1- ^{14}C)leucine, (ie. Equation 1). The total urinary N excretion over 24 hours was taken to represent 24 hour protein oxidation (E), so that values of protein synthesis (Z) and breakdown (B) could be calculated, the units being in grams of N per day. Figure 2.5 shows the plateaux achieved by ^{15}N abundance in urinary NH_3 , plasma (^{14}C)leucine specific activity, and the proportion of infused (^{14}C)leucine (and thus flux) excreted as respiratory $^{14}\text{CO}_2$ in subject J.C.W. having protein turnover measured simultaneously by (^{15}N)glycine and (1- ^{14}C)leucine methods.

Multiple (hourly) oral doses of (^{15}N)glycine

As a more convenient alternative to constant infusion, (^{15}N)glycine was given orally at an accurately known rate of about 2.0 mg ^{15}N per hour, for 14 hours. The calculation of flux, synthesis and breakdown, from plateau values of ^{15}N abundance in urinary ammonia was the same as for constant intravenous infusion techniques.

Single dose of (^{15}N)glycine (intravenous, oral)

The single dose method used has been described in detail by Waterlow et al (1978). For intravenous administration, an accurately weighed quantity of about 100 mg (^{15}N)glycine was dissolved in 22.0 ml of N-saline, sterilised by Millipore filtration, and 20.0 ml of this solution administered by syringe pump over a 1 hour period. When administered as a single oral dose, the same quantity of isotope was dissolved in

water and consumed as a bolus. Urine was collected immediately prior to administration of isotope, and used to measure the pretest abundance of ^{15}N for that subject. Initially urine was collected over 2 time intervals, 0 - 12 hours and 12 - 24 hours after the isotope was given. As the results from different methods emerged, the urine collection endpoint was changed from 12 hours to 9 hours, so collection intervals were made 3 hourly over the first 9 hours and a final '9 - 24 hour' sample.

The flux of N (Q in g N/24 hours) was calculated from the equation described by Waterlow et al (1978) (Equation 11 in Introduction):

$$Q = d / \int_0^T S_t dt = d / \bar{S} \times \frac{T}{24} \quad \text{Equation 11}$$

where d is the dose of ^{15}N given, S_t the abundance of ^{15}N in ammonia at time t , and \bar{S} the weighted mean of the ^{15}N abundances in urinary ammonia in the time urinary samples over the collection period T hours

$$(\text{i.e. } \bar{S} = \frac{\sum_{t=0}^T S_t t}{T})$$

Chemical estimations

These were routinely but meticulously done by Miss Hilda Sheppard, and included analysis of most urine samples for total N, NH_3 , creatinine and ^{15}N abundance, as described by Golden and Waterlow (1977). Total urinary nitrogen was measured by the micro-Kjeldahl method with distillation with a Markham still into 2% (w/v) borate. For NH_3 quantitation urine was made strongly alkaline and the NH_3 collected into boric acid by aeration. After titration the NH_3 was redistilled into 0.1 N HCl, and

the distillate assayed for ^{15}N abundance by the method of Sprinson and Rittenberg (1949), with a single collector MS20 mass spectrometer (AEI Scientific Apparatus Ltd, Manchester, U.K.). Samples usually contained between 0.01 and 0.2 atoms % excess of ^{15}N in urinary NH_3 .

These measurements of protein turnover, as part of a collaborative study with Professor J.C. Waterlow and Dr P.J. Garlick, were used to compare the (^{15}N)glycine/ NH_3 end product results with (1- ^{14}C)leucine results, to monitor the time-course of adaptive changes in protein turnover in response to diet, and to compare the validity of different dose-administration techniques of (^{15}N)glycine. The results are presented in Section 5.

2.3. PRESENTATION OF RESULTS

Throughout this thesis protein turnover results are usually expressed in grams of protein per 12 hours. In the literature, a number of protein-related units are used, including grams of protein, grams of protein/Kg body weight, grams of N, millimoles of amino acid, but in most cases the rates are expressed over a time interval of 24 hours. The results in this thesis show that this is mostly unsatisfactory, and results expressed in terms of 'protein-units' per day, are often far from the actual quantity of 'whole-body protein' likely to be synthesised or broken down over 24 hours. This is because most methods measure protein turnover rates over a small portion of the 24 hour day, and these rates are then assumed to be representative of 24 hour rates.

This thesis demonstrates that whole body protein synthesis is acutely sensitive to dietary intake, and hourly rates of protein synthesis depend on hourly rates of protein intake. It becomes clear that protein turnover rates measured during a feeding phase are very different from post absorptive (fasting) rates, and not at all representative of genuine rates over a normal 24 hour day.

Our experimental protocols have been designed so that the 24 hour day is divided into a 12-hour feeding phase and a 12-hour fasting (post-absorptive) phase and although this is somewhat artificial, it allowed food intake to be administered at a constant rate in 12 equal hourly portions, the constant intake being necessary for valid protein turnover measurements. The results showed remarkably different turnover rates in both these phases, indicating that protein turnover should either be expressed as an hourly rate, or as a representative rate for each of the rates for the 12-hour feeding or 12-hour fasting phases.

The 12-hour rates seemed easier to conceptualise, and hence results are usually expressed for this period. Twenty-four hour rates can then be calculated as a summation of the two different 12 hour rates.

Although results are expressed as grams of protein per 12 hours, measurements are actually made in terms of μ moles of leucine per hour for the leucine constant infusions, and grams of N per urine collection period (eg. 9 hours) for (^{15}N)glycine measurements. However, it is protein metabolism that we are fundamentally interested in, rather than leucine metabolism, so results are converted into terms of 'whole-body protein'. An accurate conversion would actually require knowledge of the leucine content, the quantity, and turnover rate of every individual body protein, clearly quite unknown at present. Thus the assumption that the factor of 8% can be used as the leucine content of total body protein (Block & Weiss, 1956) introduces an unknown error.

Because rates of flux, synthesis, breakdown and oxidation are all expressed in terms of 'whole-body protein', a discrepancy appears to arise because in these terms protein breakdown (B) does not mathematically equal the difference between flux and intake (ie. $Q - I$). This is because the leucine content of dietary protein always differed from the 8% of 'whole body protein', and was usually between 9.6% - 10.0%. The dietary protein was thus a different protein from 'whole-body protein'. Nevertheless, results were always calculated in terms of μ moles of leucine, and in those terms there was no such discrepancy, and $Q = Z + E = B + I$. Results for Q, Z, B, and E are thus presented in terms of 'whole body protein' (containing 8% leucine) whilst intake I is expressed as true dietary protein (with measured leucine content usually between 9.6% - 10.0%).

The results also show that protein turnover in adults seems to be unrelated to body weight, and no advantage is gained by expressing them in terms of grams protein/Kg body wt/12 hours. The following table (Table 2.5) shows the coefficients of variation for flux measurements in both obese and control patients.

TABLE 2.5. Coefficients of variation for mean flux rates, for a group of 10 obese patients and 5 control patients, expressed as g/day and g/Kg/day.

	% COEFFICIENT OF VARIATION FOR MEAN FLUX RATES (Q)	
	For Q in g Protein/24 hrs	For Q in g Protein/Kg/24 hrs
CONTROL GROUP	12.11 %	12.92 %
OBESE GROUP	10.28 %	13.28 %

James et al (1976) reached the same conclusion in their measurements of protein turnover in 6 normal subjects. They also expressed results as a function of lean body mass (measured by ^{40}K), and as a function of metabolic rate (uptake of O_2), but found their best coefficient of variation with results expressed in grams protein/day (see Table 2.6).

TABLE 2.6. Coefficients of variation for the mean protein synthesis rate for a group of 6 normal subjects, expressed in 4 different ways (from James et al 1976).

	FOR MEAN RATE OF PROTEIN SYNTHESIS EXPRESSED AS			
	g/day	g/Kg/day	g/kg LBM/day	Kg protein/l of O_2
% Coefficient of Variation	12.2 %	16.7 %	15.20 %	21.2 %

They therefore concluded that variations in protein synthesis rates seemed unrelated to anthropometric or physiological indices commonly used in metabolic studies, and as our measurements also confirmed this, the results presented in the following sections are whole body measurements, simply expressed as grams of protein per 12 hours.

These studies have generated a large amount of individual patient data, and the majority of these data have been tabulated in a series of Appendices at the back of the thesis. In the Results Sections the mean values for different experimental groups have been presented, as it was felt that the significance of both absolute values and relative changes in protein turnover, could best be appreciated in this form.

SECTION 3 PROTEIN INTAKE AND PROTEIN TURNOVER

RESULTS 1

DISCUSSION 1

RESULTS 1

It was the remarkable data of Sender et al (1975) that prompted the planning of this first series of protein turnover measurements. They used L-(U-¹⁴C)-tyrosine infusions to measure protein turnover in 5 obese women before and after a 3 week diet of only 300 Kcal/day of glucose syrup (1.3 MJ, 0 protein), and demonstrated a halving of the rate of protein synthesis and a 60% fall in the oxidation rate of tyrosine. They concluded that this effect was a marked adaptive process in response to restriction of both dietary protein and energy.

Although there have been many past studies investigating the effect of dietary protein and energy on nitrogen balance, relatively few have tried to measure the effect of diet on whole body protein synthesis and breakdown. Where this has been attempted, an ¹⁵N tracer has usually been used (eg. Tschudy et al, 1959; Kassenaar et al, 1960; Picou & Taylor-Roberts, 1969), and results have usually suggested that energy content, but not protein content, influenced the rate of protein synthesis. Yet the data and conclusions of Sender et al (1975) were in marked contrast to this.

It was consequently decided to examine some of the effects of dietary protein and energy on whole body protein metabolism, to ascertain whether adaption does occur in response to a prolonged period of low protein and/or low energy intake, and if so its nature, extent and timing. The suggested superiority of (1-¹⁴C)-leucine as a tracer (James et al, 1976), still largely untested at that time (apart from a study on 4 patients by O'Keefe et al, 1974), made the 1-¹⁴C-leucine infusion method the method of choice for these studies, thereby avoiding the 2 notable disadvantages of incomplete oxidation to ¹⁴CO₂ and an unknown contribution to flux from other pathways,

phenomena found to occur with (U-¹⁴C)tyrosine infusions.

It was planned to initiate this study by repeating the work of Sender et al (1975), using (l-¹⁴C)leucine, in an attempt to confirm their findings of a marked decrease in whole body protein synthesis and oxidation in response to a prolonged low energy/protein-free diet. The study would then continue using an altered dietary protocol so that results might indicate whether the low protein intake, the low energy intake or a combination of both was responsible for the protein turnover changes.

Obese patients, admitted to our metabolic beds for a period of inpatient weight reduction, were all initially maintained and stabilised for 3 days on 'Normal' Diets A or B, as detailed in Section 2 (Methods and Techniques), designed to meet energy requirements, and supply about 70 g Protein (accurately known) per day. They were then allocated into one or other of two low energy diet groups - Diet O (supplying approximately 500 Kcal (2.1 MJ) and no protein each day), or Diet P (supplying approximately 500 Kcal (2.1 MJ) and 50 g protein each day), and were kept on these diets for 3 weeks. The composition details of 'Normal' Diets A & B, Diet O and Diet P are given in Methods and Techniques section. Whole body protein turnover was measured on two occasions in each patient, initially on day 3 of the 'Normal' Diet, and finally after 3 weeks on either Diet O or Diet P, whilst still on that diet. In these measurements (l-¹⁴C)leucine was infused over 12 hours (9 am - 8 pm), and the daily dietary intake was also administered hourly over this period, in 12 equal portions.

Originally there were 5 patients allocated to Diet O group (500 Kcal/O protein for 3 weeks), but with subsequent investigation of diurnal changes in protein turnover (see next section: Results 2), a further 4 patients were added to this group. Except for a slightly

lower daily energy intake in the 3 weeks weight reduction phase (ie. 300 Kcal/day) the dietary regime used by Sender *et al* (1975) was identical to that of our 9 patients in the Diet O group. It was therefore hoped that the results from the Diet O group would confirm and quantitate the "adaptive changes", suggested by Sender *et al* (1975), in both protein synthesis and oxidation. The results from the Diet P group should then indicate whether the presence or absence of dietary protein can significantly influence rates of protein synthesis, oxidation and breakdown, at least at a low energy intake of 500 Kcal/day.

The following tables show the mean values for anthropometric features of the two groups of obese patients. The values for the individual patients are tabulated in Appendix 1.1. (Diet O group) and Appendix 1.2. (Diet P group).

TABLE 3.1. Mean values of dietary and anthropometric characteristics for patients in the Diet O group. (See Appendix 1.1.).

Mean Daily Dietary Intake and Duration		1782 Kcal/67 g Protein for 3 Days 484 Kcal/ 0 g Protein for 20 Days						
No	SEX	AGE (Yrs)	HEIGHT (cm)	INITIAL WEIGHT (Kg)	% IDEAL WT (%)	<u>WEIGHT</u> TOTAL (Kg)	<u>LOSS</u> per Day (g/day)	
<u>MEAN ± S.D.</u>	9	8F	50.7	162.7	97.9	159.8 %	6.4	321
		1M	±6.6	±5.1	±10.7	±15	±1.0	±48

TABLE 3.2. Mean values of dietary and anthropometric characteristics for patients in the Diet P group. (See Appendix 1.2.).

Mean Daily Dietary Intake and Duration		1925 Kcal/70 g Protein for 3 days 510 Kcal/48 g Protein for 20 days						
No	SEX	AGE (Yrs)	HEIGHT (cm)	INITIAL WEIGHT (Kg)	% IDEAL WT (%)	WEIGHT TOTAL (Kg)	LOSS per Day (g/day)	
<u>MEAN</u> ± <u>S.D.</u>	4	3F	49.8	165.8	122.0	181.9 %	6.5 325	
		1M	±8.1	±8.7	±37.7	±45.3	±2.4 ±120	

Although the Diet P group of obese patients appears, from its mean values, to be a little heavier than the Diet O group, this is largely because of one particularly enormous male patient (S.B. - see Appendix 1.2.). In fact the groups are well matched overall, and the t-test for sample means shows no significant difference between the two groups for age, height, initial weight, percent of ideal weight, or weight loss.

The first set of results (Table 3.3.) shows the mean values of flux (Q), synthesis (Z), breakdown (B), oxidation (E), and intake (I) for the Diet O group, all expressed in grams protein/12 hours. It should be reiterated that results are actually measured and calculated in terms of μ moles of leucine/hour, and the conversion to grams of protein assumes that a single factor (8.0%) can be used for the proportion of leucine in total body protein. This assumption is an approximation and the error it introduces is unknown. However, since leucine is used as a representative tracer for protein, the basic interest being protein metabolism rather than leucine metabolism, the results are presented

TABLE 3.2. Mean values of dietary and anthropometric characteristics for patients in the Diet P group. (See Appendix 1.2.).

Mean Daily Dietary Intake and Duration				1925 Kcal/70 g Protein for 3 days 510 Kcal/48 g Protein for 20 days				
	No	SEX	AGE (Yrs)	HEIGHT (cm)	INITIAL WEIGHT (Kg)	% IDEAL WT (%)	WEIGHT TOTAL (Kg)	LOSS per Day (g/day)
<u>MEAN</u> \pm S.D.	4	3F	49.8	165.8	122.0	181.9 %	6.5	325
		1M	± 8.1	± 8.7	± 37.7	± 45.3	± 2.4	± 120

Although the Diet P group of obese patients appears, from its mean values, to be a little heavier than the Diet O group, this is largely because of one particularly enormous male patient (S.B. - see Appendix 1.2.). In fact the groups are well matched overall, and the t-test for sample means shows no significant difference between the two groups for age, height, initial weight, percent of ideal weight, or weight loss.

The first set of results (Table 3.3.) shows the mean values of flux (Q), synthesis (Z), breakdown (R), oxidation (E), and intake (I) for the Diet O group, all expressed in grams protein/12 hours. It should be reiterated that results are actually measured and calculated in terms of μ moles of leucine/hour, and the conversion to grams of protein assumes that a single factor (8.0%) can be used for the proportion of leucine in total body protein. This assumption is an approximation and the error it introduces is unknown. However, since leucine is used as a representative tracer for protein, the basic interest being protein metabolism rather than leucine metabolism, the results are presented

in terms of protein*. Appendix 1.3. presents this set of protein turnover results for the individual patients in terms of μ moles leucine/hour, whilst Appendix 1.4. gives the individual values as grams of protein/12 hours.

TABLE 3.3. Mean (\pm S.D.) Protein Turnover Rates in 9 Obese Subjects, measured over the 12 hour feeding phase, initially on a 'Normal' Diet and then after 3 weeks on 'Diet O' (484 Kcal/O Protein).

DIETARY REGIMEN	FLUX Q	SYNTHESIS Z	BREAKDOWN B	OXIDATION E	INTAKE I
	Grams of Protein per 12 Hours				
Normal Diet (1782/67)	162.7 ± 16	120.4 ± 16	81.8* ± 19.5	42.3 ± 11.6	67.2* ± 4.5
Diet O (484/O)	81.5 ± 13	74.5 ± 12.3	81.5 ± 12.6	7.0 ± 1.3	O
	p < .001	p < .001	N.S.D.	p < .001	

The magnitude of these changes in protein turnover resulting from 3 weeks on a daily diet of 484 Kcal/O protein, can be further appreciated

* An apparent anomaly is that in grams of protein, the sum of B and I does not equal Q. This is because the concentration of leucine in the dietary protein (I) was not 8.0% (the value assumed for whole body protein and hence used for Q, Z, B, and E). Dietary leucine was measured for each of the diets, and was usually between 9.6% and 10.0% of protein by weight. However, when the results are expressed in terms of μ moles leucine (Appendix 1.3.), then $Q = Z + E = B + I$.

when expressed as percentage of the 'Normal' Diet turnover values. Appendix 1.5. gives the mean of these percentage values for each patient, whilst Table 3.4. expresses the mean group rates similarly.

TABLE 3.4. Mean Protein Turnover Rates for 9 Patients on 'Diet O', expressed as a percentage of rates on 'Normal' Diet.

DIETARY REGIMEN	FLUX Q	SYNTHESIS Z	BREAKDOWN B	OXIDATION E
Normal Diet (1782/67)	100 %	100 %	100 %	100 %
Diet O (484/0)	50 %	61.9 %	99.6 %	16.5 %
% Change	+ 49.9 %	+ 38.1 %	NO CHANGE	+ 83.5 %

These results are clearly comparable with the data of Sender et al, (1975), showing a large fall in protein synthesis and an even greater fall in the rate of protein oxidation. Particularly noteworthy is the completely unchanged rate of protein breakdown.

The immediate question then became one of whether the low energy intake or the low protein intake, or a combination of both, was responsible for these changes. The following tables (Table 3.5. and Table 3.6.) show the mean changes that occurred for the Diet P group (before and after 3 weeks on 510 Kcal/48 g Protein per day), where energy intake was similarly low to the Diet O group, but an adequate daily intake of protein was supplied. Individual patient values are given in Appendix 1.6.

TABLE 3.5. Mean (\pm S.D.) Protein Turnover Rates in 4 Obese Subjects, measured over the 12-hour feeding phase, initially on a 'Normal' Diet, and subsequently after 3 weeks on 'Diet P' (510 Kcal/48 g Protein).

DIETARY REGIMEN	FLUX Q	SYNTHESIS Z	BREAKDOWN B	OXIDATION E	INTAKE I
	Grams of Protein per 12 hours				
Normal Diet (1925/70)	176.3 ± 27	134.9 ± 28	91.8 ± 26	41.4 ± 4	70.0 ± 5
Diet P (510/48)	144.5 ± 30	115.0 ± 28	84.0 ± 30	29.5 ± 5.6	48.2 ± 0.1
	$p < .02$	$.02 < p < .05$	N.S.D.	$p < .02$	

In these absolute terms, there appears to be very little change in protein synthesis or protein breakdown, whilst the oxidation rate decreases only to an extent corresponding to the reduction in protein intake. When these values are expressed as a percentage of the 'Normal' Diet protein turnover values, as in Table 3.6., the apparently minimal percentage changes in protein synthesis and breakdown prompt the question of whether these are significant, genuine alterations of whole body protein metabolism, or simply measurement variability.

TABLE 3.6. Mean Protein Turnover Rates for 4 Obese Patients, before and after 3 weeks on 'Diet P', expressed as a percentage of rates on 'Normal' Diet. (Also see Appendix 1.7.)

DIETARY REGIMEN	FLUX Q	SYNTHESIS Z	BREAKDOWN B	OXIDATION E	INTAKE I
Normal Diet (1925/70)	100 %	100 %	100 %	100 %	100 %
Diet P (510/48)	82.0 %	85.2 %	91.5 %	71.3 %	68.9 %
% Change	+ 18.0 %	+ 14.8 %	+ 8.5 %	+ 28.7 %	+ 31.1 %

When the absolute values for Q, Z, B, and E were tested (t-test for difference between matched pairs) to determine statistically whether they had significantly decreased after 3 weeks on Diet P, flux and oxidation rates had both significantly changed ($p < .02$), as expected. However there was no statistically significant difference between the rates of protein breakdown on Normal Diet and Diet P, and although in statistical terms protein synthesis rates did appear significantly different, the calculated probability only marginally achieved this status ($.02 < p < .05$).

In fact, as many further measurements of protein turnover were completed (results shown in subsequent sections) and improved techniques rendered much better measurement precision (S.D. almost halved), it became clear that there was no real change in whole body protein synthesis, nor in breakdown, as a result of 3 weeks on Diet P, and this conclusion was verified by t-test.

In summary, the following tables show the mean values of all protein turnover measurements for obese patients on a 'Normal' Diet for 3 days, on Diet P for 3 weeks, and on Diet O for 3 weeks, the measurements being made during the 12-hour feeding phase.

TABLE 3.7. Mean (\pm S.D.) Absolute Rates of Protein Turnover, during Feeding 12 hrs, on 'Normal' Diet, Diet P, and Diet O.

DIETARY REGIMEN	No. of PATIENTS	Q	Z	B	E	I
		g of Protein/12 Hours				
Normal Diet (1712/70.5)	19	167.5 ± 17	122.9 ± 17	83.0 ± 17.5	44.6 ± 8	70.5 ± 3
Diet P (510/48)	4	144.5 ± 30	* 115.0 ± 28	* 84.0 ± 30	29.5 ± 5.6	46.2 ± 0.1
Diet O (484/0)	9	81.5 ± 13	74.5 ± 12.3	* 81.5 ± 12.6	7.0 ± 1.3	0

* N.S.D. between these values and their respective rates for 'Normal' Diet group.

TABLE 3.8. Mean Rates of Protein Turnover during feeding 12 hours, on 'Normal' Diet, Diet P, and Diet O, expressed as a percentage of 'Normal' Diet values.

DIETARY REGIMEN	No of PATIENTS	Q	Z	B	E	I
Normal Diet (1712/70.5)	19	100 %	100 %	100 %	100 %	100 %
Diet P (510/48)	4	86.3 %	93.6 %	101.2 %	66.1 %	68.4 %
Diet O (484/0)	9	48.6 %	60.6 %	98.2 %	15.7 %	0

When these values are tested to determine genuine changes in protein turnover (t-test), it is clear that flux and oxidation rates in both Diet P and Diet O groups have significantly decreased from their 'Normal' Diet values. This is only to be expected for the steady state situation, in which a change in dietary protein intake is then reflected in appropriate proportional changes in oxidation (and hence flux). On Diet O oxidation rate has fallen by 84.3% to a value of only 7.0 g Protein per 12 hours - close to obligatory protein loss.

However, it is the effect on whole body protein synthesis and breakdown that is remarkable for both Diet P and Diet O. For the Diet P group rates of protein synthesis and breakdown remained unchanged, showing no significant difference from their rates on the 'Normal' Diet. In complete contrast, the Diet O group showed a

very highly significant drop in the rate of protein synthesis ($p < .001$), a decrease of some 40% below the 'Normal' Diet synthesis rate. Yet despite this change, protein breakdown remained completely unaffected.

These results suggest that if protein intake remains adequate, then a substantial decrease in dietary energy intake to a level as low as only 500 Kcal/day (well below 40% of daily energy requirement for these obese patients), even when prolonged for 3 weeks, has no effect on whole body protein turnover.

However, the rate of protein turnover is profoundly affected at this same low level of energy intake, by the presence or absence of dietary protein, normal protein synthesis rates prevailing when adequate dietary protein is supplied, but a 40% drop in synthesis after 3 weeks of a protein free diet. In a later section (Results 3), an intermediate (below requirement) level of protein intake, still at the same low energy intake, is shown to cause a proportionally intermediate drop in protein synthesis.

It therefore seems quite probable that the rate of whole body protein turnover is markedly dependant on (and directly proportional to) the level of dietary protein intake, particularly between zero and 'requirement' levels, irrespective of energy intake. For protein intakes varying above 'requirement', protein turnover continues at its normal rate uninfluenced by energy intakes ranging from 500 - 2000 Kcal/day. It is quite possible that low energy intakes may alter protein 'requirement' levels, and very low intakes of energy (ie. below the 500 Kcal/day level of these studies) may still possibly affect the rate of protein turnover.

Protein synthesis and oxidation rates seem to be the 2 processes sensitive to dietary protein intake. Protein breakdown appears

insensitive and completely unaffected by low energy and low protein intakes, at least over prolonged periods.

DISCUSSION 1

These results are significant, because for the first time, they appear to demonstrate that protein intake itself has a substantial effect on the rate of whole body protein synthesis.

At first this appears to be in marked contrast to previous studies which have tended to show that protein synthesis is uninfluenced by varying levels of protein intake. Picou & Taylor-Roberts (1969), and Golden et al (1977) found no change in the rate of protein synthesis when energy intake was kept constant and protein intake ranged from 0.6 to 5.2 g protein/Kg/day, in studies on children recovered from malnutrition. Steffee et al (1976) showed no significant change in whole body protein synthesis when 6 normal adults on a constant, adequate energy intake changed their daily protein intake from 1.5 to 0.38 g protein/Kg/day. Both these studies tended to show an increase (not significant) in protein breakdown with the lower protein containing intakes. However, in both these studies the levels of protein intake were never below maintenance.

In our studies, we have also shown that when protein intake is varied only above maintenance levels (eg. Diet P group; 70-48 g protein/day \approx 0.61-0.42 g Protein/Kg/day) there is no effect on whole body protein synthesis rate. However, in contrast to the trend in the above 2 studies, we found no change in protein breakdown either.

The initial aim of this study was to repeat the low energy/protein free dietary protocol of Sender et al (1975b), and using (1-¹⁴C)leucine, ascertain the validity of their (U-¹⁴C)tyrosine measurements of whole body protein turnover. They infused their obese patients for 10 hours with L-(U-¹⁴C)tyrosine, and over that period fed the appropriate diet in equal aliquots at regular intervals. Although their results assume that the measured rates are representative of 24 hour protein turnover,

in fact their results are appropriate only for the period of feeding (as will be shown in Results 2). The following table (Table 3.9.) compares their results with those for the Diet O group of patients, expressing the protein turnover rates in terms of total grams of protein synthesised (or oxidised) over the appropriate 12-hour feeding period.

TABLE 3.9. Comparison of Protein Turnover Rates in Obese Patients, initially on a 'Normal' Diet and then after 3 weeks on low energy protein-free diet. Data from Sender *et al* (1975b) using L-(U-¹⁴C)tyrosine, and this study using (1-¹⁴C)leucine.

	Sender <i>et al</i> (1975b) L-(U- ¹⁴ C)tyrosine 5 patients	This study L-(1- ¹⁴ C)leucine 9 patients (Diet O group)
<u>A. NORMAL DIET</u>		
INTAKE Kcal (MJ)/Protein	2000(8.4)/84	1782(7.5)/67
(Z _N) SYNTHESIS g Protein/12 hr	200 ± 61	120.4 ± 16
(E _N) OXIDATION g Protein/12 hr	44	42.3 ± 12
<u>B. LOW ENERGY/ZERO PROTEIN DIET</u>		
INTAKE Kcal (MJ)/Protein	300(1.3)/0	484(2.0)/0
(Z _O) SYNTHESIS g Protein/12 hr	97 ± 43	74.5 ± 12
(E _O) OXIDATION g Protein/12 hr	17	7.0 ± 1
<u>PERCENT CHANGE</u>		
$\frac{(Z_N - Z_O)}{Z_N} \times 100 \%$	↓ 51.5 %	↓ 38.1 %
$\frac{(E_N - E_O)}{E_N} \times 100 \%$	↓ 61.4 %	↓ 83.5 %

Although differences in the absolute values are immediately noticeable, both studies show large and similar proportional decreases in both synthesis and oxidation, as a consequence of the prolonged simultaneous deficit of both energy and protein in the diet.

The notably higher protein synthesis rates obtained by Sender et al (1975b) as well as the larger percentage drop in synthesis and smaller percentage decrease in oxidation rate, are probably a result of their use of L-(U-¹⁴C)tyrosine, rather than the slightly lower energy intake of their obese women. Both (U-¹⁴C)tyrosine and (l-¹⁴C)leucine may underestimate synthesis rates because their intracellular specific activities are diluted by internal recycling, and tend to be lower than their measured plasma specific activities. However the disappearance of some (U-¹⁴C)tyrosine into other metabolic pathways or body stores is another source of error which tends to underestimate oxidation and overestimate synthesis rates. In addition, the de novo synthesis of tyrosine from phenylalanine, makes it difficult to estimate confidently protein breakdown (James et al, 1976).

As well as confirming the changes in protein synthesis and oxidation that Sender et al (1975b) demonstrated with prolonged combined protein and energy deficit, our study has gone on to demonstrate the unique finding, that in these obese patients, it was largely the protein deficit itself that was responsible for the 40% drop in protein synthesis. The results clearly show that when daily energy intake was only 500 Kcal (2.1 MJ) for 3 weeks, so long as protein was supplied in quantities adequate for nitrogen balance (ie. 48 g protein/day) then both protein synthesis and breakdown remained normal. Yet if no protein was supplied, the rate of protein synthesis fell to only 60% of its normal value, though protein breakdown still remained normal.

These data appear to imply that protein intake itself is the major determinant of protein synthesis rate, and that restriction of energy intake, at least to a level as low as 500 Kcal/day, has little effect on whole body protein synthesis. Marliss *et al* (1978) similarly concluded that protein supply is the principal determinant of protein sparing. Certainly protein breakdown appears completely unaffected by either protein or energy intakes, over a prolonged period.

Contrary to these findings, some previous studies have suggested a correlation between dietary energy intake and whole body protein synthesis. Sim *et al* (1979) demonstrated a substantial decrease in protein synthesis when 5 normal young men being fed intravenously, changed from a regime supplying adequate protein and energy (1.0 g amino acids/Kg and 30 Kcal/Kg of glucose) to one supplying only the protein but no additional energy (i.e. 1.0 g amino acid/Kg, only). Protein breakdown did not change.

There are a number of differences that could well account for the apparent discrepancy between the results of Sim *et al* (1979) and our results. These include the type of subject (normal versus obese), the length of time (only 6 days) on the dietary regime, different measurement methods (^{15}N -glycine versus $(1-^{14}\text{C})$ leucine), and difference in the dietary regimes. However, it seems likely that the crucial difference is one of energy intake levels as shown below.

It becomes clear, from results presented in subsequent sections, that the dynamic processes of protein metabolism are acutely sensitive to changes in dietary intake, even from hour to hour. The hourly rate of protein turnover is dependant on the hourly rate of protein intake (below 'requirement' levels). Daily intakes simply expressed as Kcal/day and grams of protein/day are often completely incomparable if these intakes have, in fact, been delivered over different time periods within the 24 hours. Sim *et al* (1979) administered their protein and

energy intakes continuously over 24 hours by intravenous infusion. In our study daily diets were administered over 12 hours, and our protein turnover measurements relate specifically to that feeding period.

When protein and energy intakes are expressed on an hourly basis it becomes clear that there is no contradiction between the results of Sim et al (1979) and our study (Table 3.10.). The energy intake of subjects in their group on amino acids only, was just 12 Kcal/hour, substantially lower than the 42 Kcal/hour for our Diet P group.

Recent data from Jeejeebhoy (1977) have further helped to clarify and indeed neatly link all 3 studies. He has shown that the negative nitrogen balance in a group of 'not immediately post-operative patients', receiving only intravenous amino acids at a rate of 1 g/Kg/day (ie. 3 g Protein, 12 Kcal per hour - the same as for Sim et al, 1979), could be changed to nitrogen balance, simply by increasing the total caloric intake up to only 14 Kcal/Kg/day with the same protein intake (ie. 3 g Protein, 41 Kcal per hour - similar to our Diet P group intake). He also showed that by increasing the level of protein intake even less energy was necessary to achieve nitrogen balance, so that at 2 g Protein/Kg/day (ie. 6 g Protein, 24 Kcal per hour - an identical protein intake to our 'Normal' Diet group) no non-protein energy was required at all for nitrogen balance.

It therefore seems probable that below a certain requirement level of protein intake, additional energy enhances the influence of protein intake in maintaining normal protein synthesis and nitrogen balance. Thus at an energy intake of 40 Kcal/hour, a protein intake of 4 g/hour maintains normal protein synthesis, 3 g/hour still maintains N-balance (Jeejeebhoy, 1977), but 2 g/hour results in a decrease in protein synthesis by some 20% (Garlick, Clugston & Waterlow, 1980),

TABLE 3.10. The effect on the relationship between protein intake and whole body protein synthesis (or N-balance), of addition of extra energy.

DAILY INTAKE Kcal/g Protein		HOURLY PROTEIN INTAKE g/hour	HOURLY ENERGY INTAKE Kcal/hour	EFFECT ON NORMAL PROTEIN SYNTHESIS/ N-BALANCE	REFERENCE
0/0	Starvation	0	0	↓ 48 % in Synthesis	(Section 5)
484/0	Diet O	0	40	↓ 40 % in Synthesis	(Section 3)
-	-	1			
500/25		2	41	↓ 20 % in Synthesis	(Section 5)
280/70	I.V. Amino Acids only	3	12	↓ 38 % in Synthesis	Sim et al, 1979
1000/70	I.V. Amino Acids +	3	41	N-Balance	Jeejeebhoy, 1977
2100/70	Energy	3	100	N-Balance	Sim et al, 1979
2900/70		3	128	+ve N Balance	Jeejeebhoy, 1977
510/48	Diet P	4	42	Normal Synthesis	(Section 3)
-	-	5			
560/144	I.V. Amino Acids only	6	24	+ve N Balance	Jeejeebhoy, 1977
1712/70	'Normal' Diet	6	142	Normal Synthesis	(Section 3)

and 0 protein results in a 40% drop in synthesis. However without the addition of the extra energy, 3 g protein/hour does not maintain N balance (Jeejeebhoy, 1977) but causes a 38% drop in synthesis (Sim et al, 1979). Table 3.10 shows these data and suggests that a series of curves could be drawn for different levels of energy intake, showing how the correlation between protein intake and protein synthesis is affected by the addition of extra energy. The state of N-balance reflects protein synthesis because on these prolonged diets breakdown appears to be unaffected.

All the protein turnover studies referred to for comparative purposes (ie. Picou & Taylor-Roberts, 1969; Golden et al, 1977; Steffee et al, 1976; and Sim et al, 1979) measured protein turnover rates with ^{15}N -glycine. It was decided to use this method on our own obese patient groups ('Normal' Diet, Diet P, and Diet O), as even further confirmatory evidence of the remarkable influence of protein intake on the rate of whole body protein synthesis, already demonstrated by the (1- ^{14}C)leucine measurements.

The following table (Table 3.11) presents the mean group values for flux, synthesis, breakdown, oxidation rate, and protein intake for 6 obese patients. Initially these obese patients were on the 'Normal' Diet described previously, then half transferred to Diet P for 3 weeks and the other half were on Diet O for 3 weeks. The ^{15}N -glycine was administered by oral hourly multiple dose technique at an accurately known dosage of about 10.0 mg ^{15}N -glycine per hour. The plateau abundance of ^{15}N in urinary NH_3 was used to calculate flux, a method developed by Waterlow et al, 1978. The results for the individual patients are given in Appendix 1.8.

TABLE 3.11. Mean (\pm S.D.) Rates of Protein Turnover, measured by multiple dose ^{15}N -glycine/ammonia end product method, for 6 obese patients initially on 'Normal' Diet, then for 3 weeks on Diet P or Diet O.

Dietary Regime	Q	Z	B	E	I
	g Protein per 12 Hours				
NORMAL DIET	198.2	158.5	136.2	39.7	62.0
	± 30	± 37	± 30	± 7	
DIET P	193.9	167.9	147.9	26.0	46.0
	± 21	± 41	± 18	± 20	± 4
DIET O	104.4	92.4	104.4	12.0	0
	± 34	± 32	± 34	± 3	

These results, in terms of statistically significant changes, are identical to the findings with $(1-^{14}\text{C})$ leucine, even though the absolute values for flux, synthesis and breakdown are all a little higher with the ^{15}N measurements. On Diet P, both protein synthesis and breakdown show no significant change from their 'Normal' Diet values. On Diet O, whole body protein synthesis decreases significantly ($p < .05$), and whilst protein breakdown appears to fall, its decrease is not statistically significant. With the results expressed as a percentage of their 'Normal' Diet values, it is clear that $(1-^{14}\text{C})$ leucine and ^{15}N glycine give excellent agreement on the magnitude of change.

TABLE 3.12. Mean rates of Protein Turnover, measured by ^{15}N -glycine/ NH_3 end product method, expressed as a percentage of 'Normal' Diet values.

DIETARY REGIMEN	Q	Z	B	E	I
NORMAL DIET	100 %	100 %	100 %	100 %	100 %
DIET P	97.8 %	105.9 %	108.5 %	65.5 %	74.2 %
DIET O	52.7 %	58.3 %	76.7 %	30.2 %	0

Having demonstrated these changes in whole body protein turnover with three different techniques (ie. L-(U- ^{14}C)tyrosine, L-(1- ^{14}C)leucine, and (^{15}N)glycine) it was felt their validity was well established and beyond reasonable doubt. Consideration then of the absolute values of whole body protein synthesis, breakdown and oxidation rates, and the possible significance of their changing in response to protein intake, immediately raised a series of fascinating questions:

- Are these particular changes a phenomenon only associated with obesity?
- If these changes are 'adaptive', how long does it take for the the protein synthesis rate to fall to the 'adapted' level?
- What happens to protein turnover in response to diurnal feeding and fasting patterns - ie. are there acute diurnal changes in protein turnover?
- The results have shown protein oxidation rate to be only half the rate of protein intake during the measurement (feeding) 12 hours Where does the other half of the intake accumulate?
- Is there an expansion of free amino acid pools during the feeding phase to accommodate the accumulating 50% of protein intake, or is there an actual expansion of body protein mass?

The actual magnitude of the difference between oxidation and intake, is best appreciated in terms of leucine rather than protein. This is because oxidation rates are measured in terms of μ moles leucine/hour, and their equivalent values in terms of protein are based on the assumption that leucine constitutes 8.0% of whole body protein (Block & Weiss, 1956). Intake was also measured as μ moles leucine/hour but leucine was found to constitute 9.6 - 10.0 % of the dietary protein fed to these patients, so in fact "whole-body protein" is not strictly comparable with dietary protein.

The following table (Table 3.13) presents the mean protein turnover rates for each of the dietary groups, with the results expressed (as measured) in terms of millimoles of leucine per hour. The results for the individual patients in Diet O group are given in Appendix 1.3.

TABLE 3.13. Mean Protein Turnover Rates, expressed as millimoles leucine/hour, for the obese patients on 'Normal' Diet, Diet P, and Diet O, measured over feeding 12 hours.

DIETARY REGIMEN (No. of Patients)	Q	Z	B	E	I
	millimoles Leucine per Hour				
NORMAL DIET (n = 19)	8.5	6.2	4.2	2.3	4.3
DIET P (n = 4)	7.4	5.9	4.3	1.5	3.1
DIET O (n = 9)	4.1	3.8	4.1	0.36	0

In this form, not only is it obvious that protein breakdown has remained quite unchanged by Diet P or Diet O, and that protein synthesis is really only significantly reduced by Diet O, but the discrepancy between rate of leucine oxidation and intake is also clear, indicating that on both 'Normal' Diet and Diet P only approximately half the incoming dietary leucine is disposed of by immediate oxidation. Does the free leucine pool expand during the 12-hour feeding phase to accommodate half the dietary leucine, or is there really a non-steady state with an increase in body protein mass? Is there a corresponding decrease in the free leucine pool, or in body protein mass, releasing leucine during the post-absorptive (ie. fasting) 12 hours, and thereby ensuring 24 hour balance?

The question of whether a diurnal expansion and contraction of the free leucine pool occurs in response to feeding and fasting is considered in the next section, where results of its actual measurement are presented and discussed.

The other obvious discrepancy is between Z and B, particularly on the two protein containing diets ('Normal' Diet $p < .001$, on Diet P $p < .01$). At first this appears simply to reflect the discrepancy between E and I, since synthesis and breakdown are calculated from the relationship $Q = Z + E = B + I$. However, if the leucine free pool remains constant (or alternatively if any change in the pool can be measured and corrected for), then any remaining discrepancy between Z and B either implies a genuinely non-steady state of the protein pool and changing body protein mass, or an incorrect estimate of the value of flux Q, since the calculation of Z, B, and E, all depend on the measured value of Q. In fact both of these alternatives are likely.

James *et al* (1976) in their measurements of protein turnover with L-(U-¹⁴C)tyrosine, found a discrepancy between tyrosine intake (27% of

flux) and tyrosine oxidation rate (20% of flux even after correction for retained $^{14}\text{CO}_2$) and interpreted this as a genuine expansion of the whole body protein pool. Indeed they calculated that the resulting difference between tyrosine uptake into protein synthesis (2.4 mmol/hour) and tyrosine release by protein breakdown (1.9 mmol/hour), was equivalent to an increase in the protein pool at a rate of 3 g protein/hour. They concluded that there must be a corresponding decrease in the mass of body protein "... at night when gluconeogenesis is occurring and amino acid oxidation continues without dietary replacement".

A very similar increase in whole body protein mass over the 12 hour feeding period is apparent for our obese patients on the protein-containing diets. For the 'Normal' Diet group the discrepancy between Z and B indicates a mean increase in whole body protein of 39.9 g/12 hours (ie. 3.3 g/hour), whilst for the Diet P group the expansion is 31.0 g protein/12 hours (ie. 2.6 g/hour). Although the difference between Z and B for patients on Diet O is small and not statistically significant, it suggests that in these obese patients, subjected to a 3 week diet of only 500 Kcal/day of glucose syrup, contraction of the body's protein pool is occurring by some 7.0 g protein in 12 hours (ie. 0.6 g/hour) during the glucose feeding phase.

There are 2 assumptions, not accounted for in the calculations above, that would affect the values of the estimated changes in protein mass. James et al (1976) assumed no expansion to occur in the free tyrosine pool during feeding, but this is probably incorrect, as there is a small but significant increase in the free leucine pool, as shown in the next section. The second assumption concerns the error involved in using plasma leucine disposal rate as an approximation for precursor pool flux.

Golden & Waterlow (1977), in their series of 30-hour (1-¹⁴C)leucine infusions on 6 continuously fed, elderly patients, were able to show that the plasma leucine specific activity method underestimated precursor pool flux by some 20%, assuming that protein oxidation and synthesis share the same precursor pool. They used a method independent of flux and based on leucine intake and urinary nitrogen excretion to estimate leucine oxidation rate, and compared these results with oxidation rates derived from ¹⁴CO₂ output and flux. By comparing these 2 estimates of leucine oxidation, they concluded from the discrepancy that precursor pool specific radioactivity was only about 80% of the plasma specific activity, a direct consequence of internal recycling in which leucine, liberated by protein breakdown, enters and dilutes the specific activity of the precursor pool and is reutilised for protein synthesis without mixing in the plasma compartment.

A similar underestimate of flux was probably operative in the measurements of flux in our obese patients on 'Normal' diet and Diet P, as both these groups were subjected to a continuous protein feeding regime over the measurement period, as with Golden & Waterlow (1977). However, their technique to obtain this correction requires an accurately known continuous dietary intake of leucine over a sufficiently long period so that simultaneous measurement of urinary nitrogen excretion, representative of protein oxidation, can be obtained. This usually requires a urinary nitrogen collection for at least 24 hours (and hence a dietary leucine intake for the same period), although measurement of changes in urea pool size may shorten the collection period. Nevertheless, by modifying their technique, the underestimate of flux was able to be calculated in patients having only 12 hours of feeding, or on Diet O with no leucine intake at all (seen in subsequent sections).

In one obese patient EE (discussed in more detail in the next section), hourly feeding was continued for some 30 hours, nitrogen balance was measured, and plasma flux was found to underestimate precursor pool flux by 21%.

TABLE 3.14. Patient EE; Estimation of Precursor Pool Flux (Q_i) and comparison with the plasma disposal rate as an estimation of flux (Q_p).

Leucine Intake I_L mmol/day	N-Intake I_N g/day	N-Excretion E_N g/day	Leucine Oxidised $E_L = I_L \times \frac{E_N}{I_N}$ mmol/day	Leucine Oxidised (as % of Flux - from $^{14}\text{CO}_2$ output)
55.18	11.73	8.15	38.34	24.2 %

<u>Precursor Pool Flux</u> $Q_i (= E_L \times \frac{100}{24.2})$ mmol Leu/day	<u>Plasma 'Flux'</u> Q_p mmol Leu/day	$\frac{Q_p}{Q_i} \times 100 \%$
158.4	125.0	79 %

Any such underestimate of flux will affect the derived value of protein breakdown rate more than protein synthesis rate, and consequently any correction for the underestimate of flux will alter the calculated value of net protein synthesis or breakdown differently (see Sections 4 and 7). The following table (Table 3.15) shows the mean estimated increase in body protein mass over 12 hours of feeding, for obese patients in the

'Normal' Diet group and Diet P group, after the measured value of flux has been corrected for an assumed 20% underestimate of precursor pool flux.

TABLE 3.15. The mean increase in body protein mass, occurring over a 12 hours feeding period, for obese patients on either the 'Normal' Diet or Diet P.

	INCREASE IN BODY PROTEIN MASS	
	g/hour	g/12 hours
NORMAL DIET GROUP	2.4	28.7
DIET P GROUP	2.0	23.6

The issues that have emerged from this first group of results, polarise into 2 distinct areas for further investigation - the acute diurnal changes in protein metabolism, and the long term 'adaptive' changes.

These results have shown that during the daytime feeding 12 hours, whole body protein synthesis, breakdown and oxidation occur at such rates as to produce a progressive state of protein imbalance. Half the dietary intake of protein is stored rather than being immediately oxidised, there is an expansion of the body's protein mass, a possible expansion of the plasma free leucine pool, and a relationship between plasma and precursor pools such that plasma measurements underestimate flux by 20%. Protein metabolism must subsequently readjust if 24-hour protein balance is to be achieved. How values of synthesis, breakdown and oxidation change, whether the free leucine pool contracts, whether these values and phenomena of protein metabolism are features

peculiar to obesity, and whether circadian rhythms rather than food intake produce these diurnal changes - these are the immediate and outstanding questions that determined the protein turnover measurements presented in the next section (Results 2).

The results have also shown remarkable changes in whole body protein synthesis and oxidation resulting from a 3 week period of low energy/zero protein diet. Once the validity of these changes was established, the questions of how much change occurred as an acute response, how much was an adaptive response, and how long it took for any such adaption to occur, were felt to merit investigation.

Investigation of these two distinct areas of the dynamics of whole body protein metabolism, required the use of different methods and techniques for protein turnover measurement. Prolonged infusions with (1-¹⁴C)leucine extending over 24 hours or longer, and combined with continuous energy expenditure measurements, were used to investigate the acute diurnal aspects of metabolism outlined above. This work is presented in the next section (Section 4).

The necessity to make repeated measurements of protein turnover at short intervals, to investigate the dynamics of any adaptive changes, precluded the use of a radioactive tracer such as (1-¹⁴C)leucine. The excreted end-product method, using the stable isotope ¹⁵N-glycine with measurement of the abundance of ¹⁵N in urinary ammonia, was therefore used, in conjunction with a programme to develop and validate this technique (J.C. Waterlow & P.J. Garlick). The results of these measurements are presented in Section 5.

SECTION 4 ACUTE AND DIURNAL CHANGES IN PROTEIN TURNOVER

RESULTS 2

DISCUSSION 2

RESULTS 2

The previous section's results clearly imply that diurnal changes in protein metabolism must occur, at least in obese subjects on a normal daytime feeding/night-time fasting regimen. During the feeding 12 hours these subjects actually increased their body protein mass to accommodate half the dietary protein intake and so during this phase whole body protein synthesis was consistently operating at a greater rate than protein breakdown. Yet if these subjects were in nitrogen balance over 24 hours, then during their fasting 12-hours an equivalent but opposite discrepancy between synthesis and breakdown must have occurred. James et al (1976) also concluded from their (U-¹⁴C)tyrosine studies that body protein mass declines at night.

Up to the present time diurnal changes in whole body protein synthesis, breakdown, and oxidation have remained a completely unexplained area of protein metabolism. Whole body protein turnover has usually been thought of (and expressed) in terms of a constant daily rate, largely because of methodological limitations - some of the methods still most widely used (eg. (¹⁵N)glycine/urea end product: Picou & Taylor-Roberts, 1969) take 2 - 3 days to complete a measurement, and the slow turnover of the urea pool also prevents the achievement of measurable nitrogen balance in less than 24 hours in adults experiencing normal daily phases of feeding and fasting. Even Sender et al (1975) believed that the rates of whole body protein synthesis which they measured represented daily rates, and they concluded that the halving of protein synthesis and 60% fall in oxidation which resulted from the 3 weeks of protein-free diet were longer term "adaptive" changes. Yet it seems quite possible that if protein turnover were acutely sensitive to protein intake, then a significant part of the

drop in synthesis and oxidation, seen after 3 weeks on a protein-free diet, might occur within hours of cessation of protein intake - ie. even during normal night-time fasting.

A group of 6 obese patients were admitted to our metabolic beds, specifically for the purpose of investigating their diurnal values of whole body protein turnover ('Diurnal' group, as shown in Section 2, Methods & Techniques). In addition another 4 obese patients ('Diet 0-Diurnal' group) undertook the same protocol before embarking on a weight reducing course. Appendix 2.1 contains the individual protein and energy intakes and anthropometric data for each patient, but the following table shows the mean values for this group of 10 obese patients.

TABLE 4.1. Mean (\pm S.D.) dietary and anthropometric characteristics for the 10 obese patients ('Diurnal' + 'Diet 0-Diurnal' group) measured for their protein turnover rates over 24 hours.

MEAN INTAKE		}	1607 \pm 291 Kcal			
(over Feeding 12-hours)			72.6 \pm 4.3 g Protein			
No	SEX	AGE	HEIGHT	WEIGHT	% of IDEAL WEIGHT	
		(yrs)	(cm)	(Kg)	(%)	
MEAN	10	10F	50.3	160.7	96.6	163.5 %
\pm S.D.			± 7.7	± 5.3	± 14.2	± 29.6

These 10 patients were initially given a high protein/weight maintenance diet ('Normal' Diet) for 3 days, their individual energy requirements being measured as described previously. On the third day their whole body protein turnover was measured for at least

24 hours, by constant infusion with (1-¹⁴C)leucine, thereby covering the feeding 12-hour period (9 am - 8 pm), and the night-time fasting 12-hour period (9 pm - 8 am).

The results from this group show unequivocally the changes in whole body protein synthesis, breakdown, oxidation, and flux, that occur in the course of a 24 hour period, when daytime feeding 12-hours changes to night-time fasting. Table 4.2 gives the mean values for the whole group. (Appendices 2.2 and 2.3 tabulate the data for individual patients in g Protein/12 hours and mmol Leucine/hour).

TABLE 4.2. Mean (\pm S.D.) protein turnover rates for the group of 10 obese patients on 'Normal' Diet, showing rates during Feeding Phase, Fasting Phase, and total 24 hour protein turnover.

PHASE	Q	Z	B	E	I
	g Protein per 12 Hours				
FEEDING	166.0	119.4	78.1	46.6	72.6
12 HOURS	± 17	± 15	± 17	± 7	± 4
FASTING	106.3	87.6	106.3	18.7	0
12 HOURS	± 18	± 16	± 18	± 4	
TOTAL	272.3	207.0	184.4	65.3	72.6
24 HOURS	± 28	± 26	± 26	± 7	± 4

When these changes are statistically tested by matched-pair t-test, they are all highly significant, leaving no doubt that the 27% decrease in whole body protein synthesis and the 36% increase in breakdown are genuine changes.

	Q	Z	B	E
Matched-pair t-test	p<.001	p<.001	p<.01	p<.001

The magnitude of these changes can be further appreciated when expressed as the percentage change that has occurred in passing from the feeding to fasting phase. Here the results are related to the feeding phase values, but later when absolute values under all conditions are considered and the fasting phase is considered as basal, values are then expressed as a percentage of the normal fasting phase values.

TABLE 4.3. Mean rates of protein turnover for the group of 10 obese patients on 'Normal' Diet, with the Fasting Phase expressed as a percentage of Feeding Phase values.

PHASE	Q	Z	B	E
FEEDING PHASE	100 %	100 %	100 %	100 %
FASTING PHASE	64.0 %	73.4 %	136.1 %	40.1 %
% CHANGE	+36.0 %	+26.6 %	+36.1 %	+59.9 %

Three remarkable facts are immediately obvious from these results:-

- (1) There was a 38% drop in protein synthesis over the 3 week period on the protein free Diet O (see Results 1) yet these diurnal results surprisingly show that over 2/3 of that drop occurs overnight, apparently routinely.

- (2) Protein breakdown has increased by 36% during overnight fasting - yet low protein and energy diets prolonged over 3 weeks (see Results 1) left protein breakdown unaffected.
- (3) If 24-hour protein synthesis, breakdown, and oxidation rates are calculated only from feeding phase measurements, they will be very different from the genuine 24 hour rates which normally result from both feeding and fasting phase protein turnover.

These points are analysed further in Discussion 2 where the absolute values of protein turnover in the feeding and fasting phases are discussed in detail, and the implications and significance of the diurnal changes, are considered. It should also be reiterated that the apparent feeding phase discrepancy such that $Q \neq B + I$ only occurs because there are different leucine concentrations in 'whole body protein' and dietary protein, and when values are expressed in terms of millimoles of leucine (as in Discussion 2) this discrepancy vanishes.

The results show that for obese women, on an adequate food intake and having the usual intake pattern of daytime feeding and night-time fasting, when the change is made from feeding to fasting phases whole body protein synthesis decreases while protein breakdown increases. Moreover, the discrepancy between synthesis and breakdown during the feeding phase which was previously shown to maintain a greater rate of synthesis than breakdown, now becomes completely reversed so that breakdown rate is constantly greater than synthesis rate during night-time fasting.

Two questions concerning the nature and cause of these changes, are particularly outstanding in their demand for investigation:

- (1) Are these diurnal changes a consequence of obesity?
- (2) Are these diurnal changes simply natural circadian rhythms?

To answer the first question, a study was set up to measure 24-hour protein turnover in a group of non-obese adults, both male and female, and this group of 5 subjects was called the 'Control group' (see Section 2). It was anticipated that results from the Control group would not only indicate whether diurnal changes in protein metabolism are a universal human phenomenon, but would also answer the question raised in the previous section (Section 3), - are there any differences in the absolute values of protein synthesis, breakdown and oxidation, between obese and normal people?

The Control group subjects were Departmental colleagues or their relatives, and their experimental protocol is outlined in the Methods & Techniques section. Table 4.4 shows the Control group's mean dietary and anthropometric characteristics, while their individual data are tabulated in Appendix 2.4.

TABLE 4.4. Mean (\pm S.D.) dietary and anthropometric characteristics for the 5 normal subjects in the 'Control' group.

MEAN INTAKE (over Feeding 12-hours)			} 1797 \pm 440 Kcal 67.2 \pm 2.9 g Protein			
No	SEX	AGE (Yrs)		HEIGHT (cm)	WEIGHT (Kg)	% of IDEAL WEIGHT (%)
Mean	5	2F	56.4	172	67.0	96.2
\pm S.D.		3M	± 9	± 4.2	± 12.8	± 16

As with the obese Diurnal groups, the Control group subjects consumed their food intake in 12 equal hourly portions over the daytime (9 am - 8 pm) feeding phase. Protein turnover measurement continued throughout the entire 24 hour period covering both daytime feeding and night-time fasting 12-hour periods. The following table (Table 4.5) presents the mean protein turnover measurements for these two phases for the Control group, expressed in grams of protein per 12 hours, and the 24 hour rates, calculated as a summation of feeding phase and fasting phase rates. (The values for individual subjects, expressed in both grams of protein and millimoles of leucine are tabulated in Appendices 2.5 and 2.6.)

TABLE 4.5. Mean (\pm S.D.) protein turnover rates for the Control group of 5 subjects, measured over 24 hours. Expressed in g Protein per 12 hours.

PHASE	Q	Z	B	E	I
	g Protein per 12 Hours				
FEEDING 12 HOURS	155.3 ± 17	112.2 ± 24.5	75.1 ± 19	43.1 ± 7.5	67.2 ± 2.9
FASTING 12 HOURS	100.5 ± 15	81.6 ± 15	100.5 ± 15	18.9 ± 4	0
TOTAL 24 HOURS	255.8 ± 31	193.8 ± 38	175.6 ± 32	62.0 ± 10	67.2 ± 2.9

When these protein turnover values are expressed as a percentage of feeding phase values (Table 4.6) to show the relative changes that occurred as a result of passing from daytime feeding to night-time fasting, once again large diurnal swings in protein synthesis, breakdown

and oxidation rates are apparent.

TABLE 4.6. Mean protein turnover rates for the Control group of 5 subjects measured over 24 hours, expressed as percentages of Feeding Phase values.

PHASE	Q	Z	B	E
FEEDING 12 HOURS	100 %	100 %	100 %	100 %
FASTING 12 HOURS	64.7 %	72.7 %	133.8 %	43.9 %
% CHANGE	+35.3 %	+27.3 %	+33.8 %	+56.1 %

Not only is it clear that the magnitude of the diurnal changes in whole body protein synthesis and breakdown is almost identical in obese and non-obese subjects, but the diurnal change in protein oxidation rates is also the same for obese and non-obese people. Furthermore, there is no statistically significant difference in absolute values of protein turnover in the feeding, fasting, or 24-hour phases, between obese and non-obese people, although the Control group means are all marginally less than obese group means. This is expressed visually in Figure 4.1, whilst it is Table 4.7 that shows the results of statistical comparison by t-test for differences between obese and Control groups in all 3 phases (the aim of the subtle message of this table being to reinforce the finding that fat and thin humanity share equality in terms of protein turnover!).

FIGURE 4.1. Diurnal changes in Protein Synthesis, Breakdown and Oxidation, in obese and non-obese subjects.

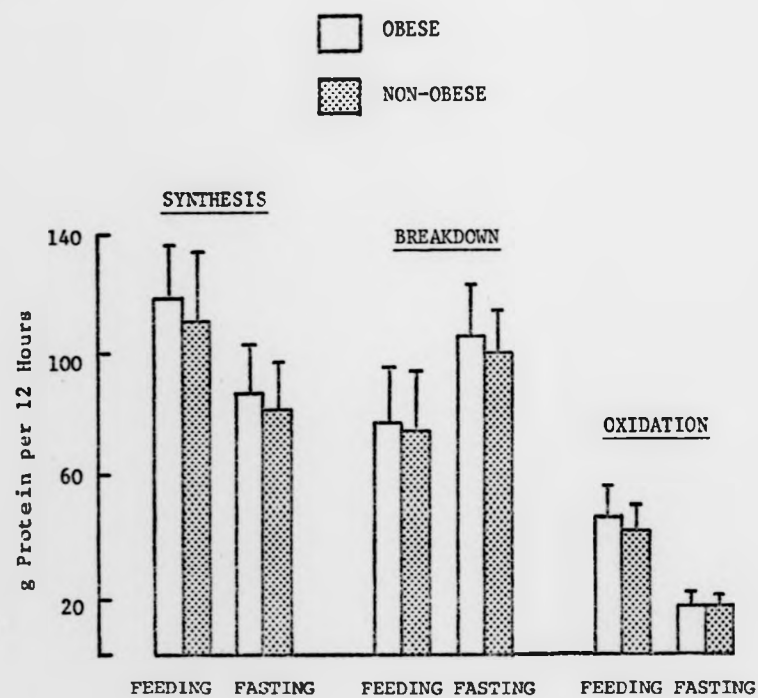


TABLE 4.7. Comparison of mean protein turnover values between Control group and the group of 10 obese patients, in Feeding, Fasting, and 24-Hour Phases (t-test for difference of means).

	Q	Z	B	E
FEEDING PHASE	N.S.D.	N.S.D.	N.S.D.	N.S.D.
FASTING PHASE	N.S.D.	N.S.D.	N.S.D.	N.S.D.
24 HOUR TOTAL	N.S.D.	N.S.D.	N.S.D.	N.S.D.

These results suggest quite conclusively that protein synthesis, breakdown, and oxidation rates are much the same in obese and non-obese adults, and that the diurnal changes that occur in protein turnover occur equally in obese and non-obese adults (Fig 4.1).

The possibility that these diurnal changes in whole body protein turnover are simply day/night natural circadian rhythms, was the second question provoking obvious attention. To settle this question, 3 studies were planned and implemented. In the first study protein turnover was measured for 18 hours in a patient being continuously fed (for 24 hours). In the second study patients having no protein intake over a 24 hour period were then refed protein, with protein turnover being measured continuously over 36 hours. Thirdly, to ensure that the infusion technique itself was not contributing to the diurnal changes, 24 hour protein turnover measurements in both an obese and a Control subject were commenced in the night-time fasting phase, and continued over 24 hours.

It was an obese female patient of 54 years (E.E) who endured hourly feeding for 24 hours whilst her protein turnover was continuously measured by (1-¹⁴C) leucine infusion. Her 24 hour intake was 1,870 Kcal (7.8 MJ) and 72.3 g protein, an intake typical of the 'Normal' Diet category - but because this quantity was consumed over 24 hours rather than the usual 12 hours, her rate of protein intake was only 3.0 g protein/hour, just half the rate of the other obese patients on 'Normal' Diet.

Table 4.8 shows her mean hourly protein turnover rates in each of 3 consecutive 6 hour periods. All other obese patients on the 'Normal' Diet feeding/fasting regimen, without exception showed large decreases in Z and E between 6 pm - 12 MN, constant low plateaux between 1 am - 6 am, followed by a large increase between 7 am - 12 MD returning Z and E back to normal high daytime values. In distinct contrast E.E showed no change whatsoever in Q, Z, B, or E over the whole 18 hour period, and this finding is supported statistically by the t-test which shows no significant difference in protein turnover between any of the 3 phases. Figure 4.2 (a) shows her individual hourly synthesis rates (Z), which fall on the regression line -

$$Z \text{ (nmol leu)} = 4.6 - 0.04t$$

The slight negative slope probably results from recycling of label from protein stores.

TABLE 4.8. Mean (\pm S.D.) hourly rates of protein turnover in obese patient E.E, during three 6-hour phases, on a constant hourly intake.

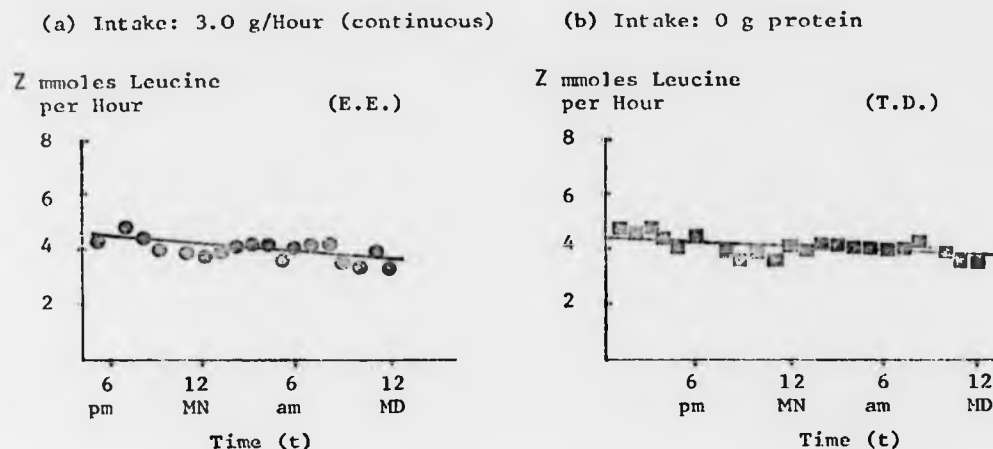
PHASE	Q	Z	B	E	I
	g Protein per Hour				
6 pm - 12 MN	8.6 ± 0.6 (N.S.D)	6.6 ± 0.5 (N.S.D)	5.0 ± 0.6 (N.S.D)	2.0 ± 0.2 (N.S.D)	3.0
12 MN - 6 am	8.6 ± 0.6 (N.S.D)	6.6 ± 0.4 (N.S.D)	5.0 ± 0.6 (N.S.D)	2.0 ± 0.3 (N.S.D)	3.0
6 am - 12 MD	8.4 ± 0.7	6.2 ± 0.6	4.7 ± 1.4	2.2 ± 0.1	3.0

These results support the more extensive measurements of protein turnover in geriatric patients made by Golden & Waterlow (1977), who also found no change in protein synthesis, breakdown, or oxidation whilst continuous nasogastric feeding was administered over a period of 30 hours.

These data alone are convincing evidence that there are no intrinsic circadian rhythms of protein turnover. Rather the presence or absence of food intake appear responsible for the diurnal rhythms in protein synthesis, breakdown, and oxidation.

The second study is discussed in detail in Section 6 (Results 4) where diurnal changes and adaption are further investigated. However, the results show that when obese patients were maintained on a low energy/protein-free diet for 3 weeks, not only did protein synthesis and oxidation remain at very low constant plateau values for as long as protein intake remained zero, but on reintroduction of dietary protein, immediate changes in protein turnover ensued. Figure 4.2 (b) shows

FIGURE 4.2. Whole-body protein synthesis, measured hourly, (a) in patient E.E. on a continuous hourly intake of protein; (b) in patient T.D. on a continuous protein free diet.



PROTEIN SYNTHESIS
mmoles Leucine
per Hour

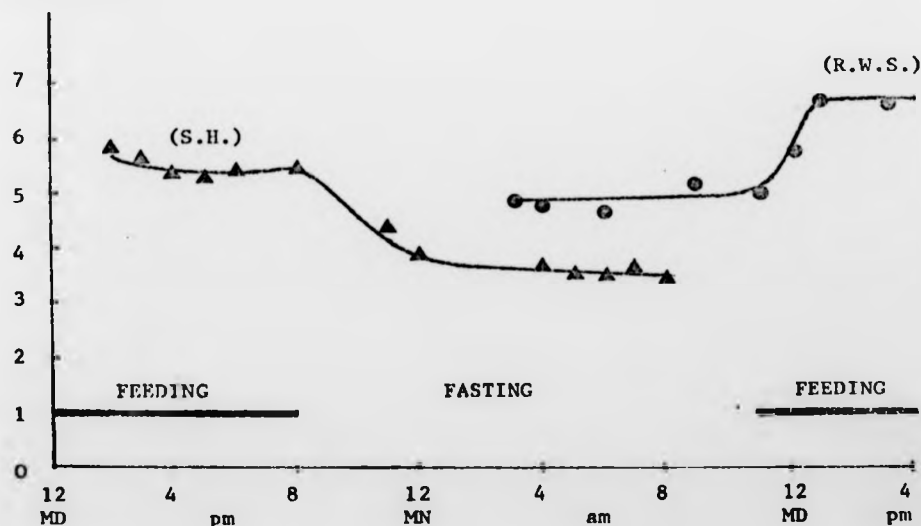


FIGURE 4.2.c. Diurnal changes in whole-body protein synthesis, related to protein feeding and fasting.

the individual hourly synthesis rates (Z) for obese patient T.D. who remained on a protein intake of zero over 30 hours. These values create the regression line -

$$Z \text{ (mmol leu)} = 4.5 - 0.03t$$

and once again its slight negative slope probably results from re-entry of (^{14}C)leucine from protein stores into the plasma leucine pool.

To ensure that the observed diurnal changes in protein turnover were not an infusion artefact, 2 patients had their 24-hour ($1\text{-}^{14}\text{C}$)leucine infusions commenced at the beginning of the night-time fasting phase and continued throughout the feeding phase on the following day (see Table 4.9). As expected, immediately the feeding phase was recommenced after night fasting, the low fasting values of protein synthesis and oxidation rapidly increased, and protein breakdown fell, indicating the acute sensitivity of protein turnover to protein intake. Figure 4.2 (c) shows the normal diurnal changes in whole body protein synthesis in an obese patient (S.H) and 'Control' subject R.W.S), both on the biphasic 'Normal' Diet regimen (12 hours feeding/12 hours fasting) but with the ($1\text{-}^{14}\text{C}$)leucine infusion beginning in the feeding phase for S.H. and in the fasting phase for R.W.S.

Table 4.9 gives the mean protein turnover values for the 2 subjects in whom ($1\text{-}^{14}\text{C}$)leucine infusions were commenced with the night-time fasting (ie. 'post-absorptive') phase. It is clear from these results, that the diurnal protein turnover changes are a consequence of the feeding and fasting phases themselves, and that it makes no difference to the values which phase is measured first.

TABLE 4.9. Mean protein turnover rates for 2 subjects (one obese female, the other a Control group male), on 'Normal' Diet regimen. The (1-¹⁴C)leucine infusions were commenced at the beginning of night-time fasting 12 hours.

PHASE	Q	Z	B	E	I
	g Protein per 12 Hours				
FASTING 12 HOURS (9 pm - 8 am)	109.1 ± 5	90.5 ± 7	109.1 ± 5	18.6 ± 2	0
FEEDING 12 HOURS (9 am - 8 pm)	144.6 ±24.5	104.5 ±20	57.7 ±25.8	40.1 ± 4.5	72.3

It has been shown that protein turnover remains constant, both day and night, if (a) there is continuous intake of protein, (b) protein intake remains continuously at zero, whilst normal diurnal changes were shown to coincide with the commencement or cessation of protein intake. From these results, it can reasonably be concluded that protein turnover has no intrinsic circadian rhythm of its own. However there are substantial diurnal changes in protein synthesis, breakdown, and oxidation rates, but these appear to occur as an acute response to dietary protein intake.

DISCUSSION 2

A number of exciting, and hitherto unknown characteristics of human whole body protein metabolism have been presented in Results 2, and perhaps the three most obvious of these are summarised below.

1. There are large diurnal changes in the rates of protein synthesis, breakdown and oxidation, and it appears that these are acutely responsive to changes in protein intake.
2. There are no differences in protein turnover rates between obese and non-obese humans on an adequate intake. Neither the absolute values nor the relative changes in protein synthesis, breakdown or oxidation that occur with feeding or post-absorptive fasting, are any different in lean and obese adults.
3. There are no natural circadian rhythms per se in whole body protein turnover.

The previous section (Section 3) showed that protein turnover was significantly affected by prolonged low protein intakes, and that the absolute values of protein synthesis, breakdown, and oxidation during the feeding phase of a normal diet were such that some diurnal change had to occur to maintain balance. Yet it was never suspected that such large diurnal changes as those shown (Results 2) might occur, particularly in protein breakdown which appeared unmoved by drastic long term changes in protein and energy intakes.

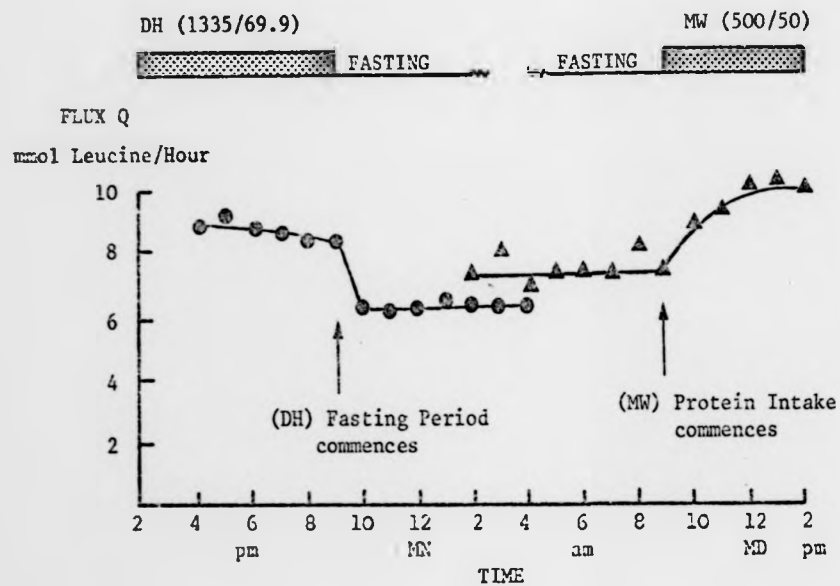
The dramatic 40% drop in protein synthesis and 80% drop in oxidation, shown to have resulted from 3 weeks of low energy protein-free diet

(Diet O), were interpreted as being adaptive changes. However the 24-hour protein turnover measurements in this section have clearly shown that when human adults on a normal dietary intake simply change to a post-absorptive state, as is usual for most people every night, then within hours whole body protein synthesis falls by some 27% and remains at that level, protein oxidation drops by about 60% and the rate of protein breakdown appears to increase by some 35%. Over 2/3 of the change that resulted from prolonged protein free dieting, has occurred overnight, even in normal people on a normal intake simply moving into their customary overnight fasting period.

The evidence strongly suggests that protein turnover rates are acutely dependent on protein intake, and acute changes in the rate of protein intake immediately produce changes in the rate of protein turnover. Intrinsic circadian rhythms were shown to play no part in diurnal protein turnover changes, and changing levels of energy intake are unlikely to be of any great influence, other than perhaps modifying the protein turnover-protein intake relationship. This was suggested in the previous section (Results 1) where it was shown that a large drop in energy intake, with protein intake remaining adequate, left protein turnover unchanged.

The extreme sensitivity of protein turnover to acute changes in protein intake can be seen from previous figures (Figs. 2.1, 2.5, and 4.2) and from Section 6 where patients on Diet O for 3 weeks suddenly have protein reintroduced to their low energy diets. Figure 4.3 shows that within one hour of ceasing or recommencing protein intake, whole-body leucine flux is already rapidly changing.

FIGURE 4.3. The rapid change in flux Q (mmol leu/hr) as an acute response to the cessation (D.H.) or commencement (M.W.) of protein intake. Intake values are given (Kcal/g Protein).



The significance of the absolute values and their imbalance is probably better appreciated when expressed in terms of leucine, especially because intake (I) is then in comparable terms with Q, Z, B, and E. Table 4.10 gives the mean group values for the 10 obese patients whose protein turnover was measured over 24 hours to investigate diurnal changes, and the results are expressed as millimoles of leucine per hour.

TABLE 4.10. Mean (\pm S.D.) values of protein turnover, in millimoles Leucine per 12 Hours, for the 10 obese patients ('Diurnal' + 'Diet-0-Diurnal' groups) measured over 24 hours with (1- 14 C)leucine.

PHASE	Q	Z	B	E	I
	millimoles of Leucine per 12 Hours				
FEEDING	101.2	72.8	47.6	28.4	53.6
12 HOURS	± 10.3	± 9.0	± 9.8	± 4.4	± 3.5
FASTING	64.8	53.4	64.8	11.4	0
12 HOURS	± 11.2	± 9.5	± 11.2	± 2.5	
TOTAL	166.0	126.2	112.4	39.8	53.6
24 HOURS	± 17.0	± 16.1	± 16.4	± 4.8	± 3.5

There are substantial leucine imbalances, not only in feeding and fasting phases, but also over the entire 24 hour period when leucine balance might have been expected. The data in Table 4.10 show an accumulation of 25.2 mmol leucine in the body during feeding phase, the same imbalance that was found with Diet O and Diet P groups on normal feeding. There is a negative balance and hence loss from the body of 11.4 mmol of leucine during the fasting 12 hours, and no over

the complete 24 hour period there appears to be a net *accumulation* of 13.8 mmol of leucine somewhere in the body.

However there are several factors that influence the magnitude and significance of the above data. These include:

- (1) Any diurnal change in the plasma free leucine pool.
- (2) The correction factor in feeding and fasting phases for the plasma method's underestimation of flux.
- (3) 24 hour N-balance.

and the effect each of these has on the results, merits analysis.

It has been proposed by some authors in the past (Adam & Oswald, 1977) that sleep is a time of anabolic restoration of tissue, and of *increased* protein synthesis. They claim that plasma and intracellular amino acid levels are unrelated, that plasma amino acids vary independently of protein intake, and the "intracellular amino acids are highest while food intake is least" (Adam & Oswald, 1980). Their implication is that plasma amino acid levels, by fluctuating independently of both protein intake and intracellular levels, might decrease so much that the specific activity of a tracer would greatly increase and thus falsely indicate that protein flux and synthesis had decreased.

However, in a number of our obese and normal subjects, plasma leucine concentrations were measured throughout feeding and fasting phases, and the effect of variations in protein intake on plasma free leucine pool measured. The results in Table 4.11 show that plasma leucine concentration is significantly affected by protein intake, in both obese and non-obese subjects, the high plasma concentrations corresponding to the feeding phase, and low plasma concentrations during fasting.

TABLE 4.11. Diurnal changes in plasma free leucine pool for 2 obese patients (E.K. and R.O.) and one 'Control' subject (J.K.G.), on the 'Normal' Diet regimen with feeding and fasting 12 hour phases.

NAME	FEEDING 12 Hrs (I) μ moles Leucine/litre plasma	FASTING 12 Hrs (II)	% CHANGE (I-II)/I \times 100 %
EK	148.7 \pm 22.2	109.4 \pm 9.0	+26.4 %
RO	148.1 \pm 19.4	100.0 \pm 3.8	+32.5 %
MEAN (OBESE)	148.4 \pm 19.6	105.0 \pm 8.4	+29.4 %
JKG (CONTROL)	101.6 \pm 10.3	71.2 \pm 5.3	+29.9 %

These results show a consistent drop of some 30 - 40 μ moles/litre in the plasma free leucine concentration, from the daytime feeding phase to the night-time fasting phase. It appears the changes in plasma leucine are dependent and sensitive to protein intake, as they vary directly with this. Further evidence follows.

Table 4.12' then shows how constant the plasma free leucine pool remains, when the feeding/fasting pattern is eliminated, by continuous (hourly) feeding of a normal total daily intake throughout the 24 hours. The result is elimination of the diurnal fluctuations of the free leucine pool.

TABLE 4.12. The effect on the plasma free leucine pool, of feeding the normal daily intake (1870 Kcal/72 g Protein) in equal hourly aliquots over the 24 hour period. (Patient E.E. Plasmas were taken for the last 18 of the 24 hours.)

	<u>TIME PERIOD</u>		
	6 pm - 12 MN	12 MN - 6 am	6 am - 12 MD
MEAN (\pm S.D.)			
Plasma free leucine	144.0	140.7	142.3
(μ moles/litre plasma)	± 10.7	± 9.8	± 5.1

The data from Tables 4.11 and 4.12 are combined in Figure 4.4 where it can be appreciated even more clearly, that the plasma free leucine pool varies directly with protein intake.

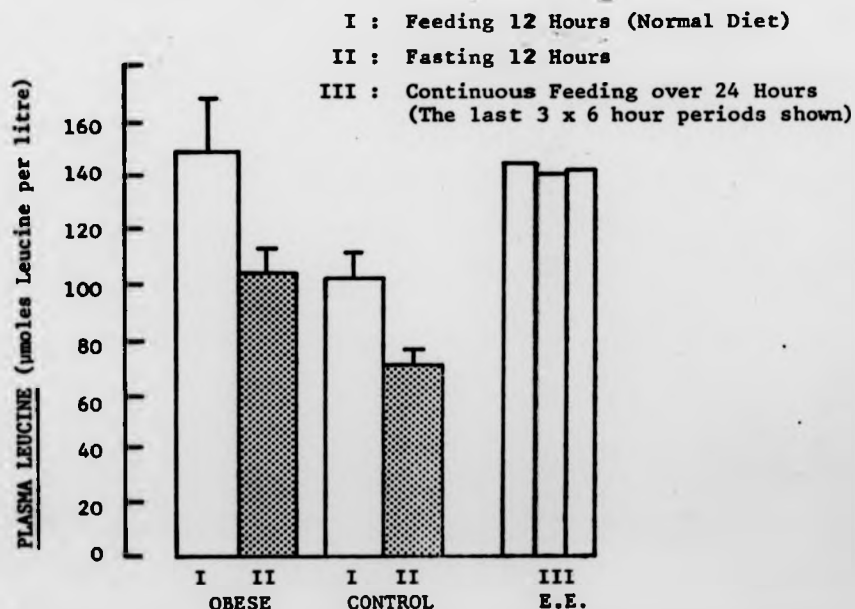


FIGURE 4.4. Plasma free leucine concentrations over 24 hours, in obese and non-obese subjects, and under different food intake situations.

If the fasting drop in plasma leucine concentration were large enough, and was reflected by the behaviour of intracellular free leucine, then it could possibly account for our claimed decrease in protein synthesis with fasting. The data from Table 4.11 show a drop of 30-- 40 μ moles per litre leucine in the plasma free leucine pool. There is no evidence to suggest that intracellular leucine does not behave similarly. Indeed recent data comparing plasma with intracellular (muscle) free leucine concentrations (Möller *et al*, 1980; Vinnars *et al*, 1975) have shown that although intracellular free leucine concentration is a little higher than plasma, similar changes occur in both compartments as a result of surgery and aging.

Assuming that a decrease of about 40 μ moles per litre leucine occurs in both intracellular and extracellular compartments as a result of post-absorptive fasting, and using the following body fluid data from Elkinton & Danowski (1955), applicable to a 70 Kg man:

Plasma H ₂ O	2.4 L
Total extracellular H ₂ O	11.5 L
Intracellular H ₂ O	31.5 L
Total Body H ₂ O	43.0 L

then the decrease in total body free leucine, as a result of fasting, would amount to 1.8 millimoles of leucine.

However, our data from Table 4.10 and Appendix 2.6, show that both non-obese and obese subjects decrease their whole body protein synthesis by 20 millimoles of leucine as a result of fasting. Clearly, any error introduced because of feeding/fasting-induced changes in the free leucine pool, leading to an overestimate of the drop in protein synthesis during fasting, will be less than 10%. It is equally clear that there would have to be an enormous and probably impossible decrease

(eg. some 500 μ moles Leucine per litre) in the body's free leucine pool, if this alone were to explain the decrease we estimated to occur in protein synthesis.

As discussed in the previous section, the (1-¹⁴C)leucine infusion method significantly underestimates flux, because of internal recycling and Golden & Waterlow (1977) estimated that in elderly patients under constant feeding conditions the underestimate was 20%. The calculation of this underestimate is based on the assumption that protein synthesis operates from the same metabolic pool as oxidation, so that the measured underestimate in oxidation then becomes applicable to synthesis.

It is worth estimating this error in these diurnal patients since fasting conditions may affect its values, and calculation of changes in body protein mass may be more accurate with the corrected values of Z and B.

Two approaches are used here. Firstly, an estimate of the error in calculating flux over the entire 24 hour period (feeding + fasting) can be made by comparing the leucine oxidation rate E_L calculated from flux and expired ¹⁴CO₂, with the leucine oxidation rate E_U calculated from urinary-N excretion. For this latter oxidation rate (E_U) it is assumed that the proportion of N derived from leucine oxidation should be the same as the proportion of leucine in dietary protein as far as N-balance, but then any excess N (and hence leucine) excreted should come from 'whole body protein'. The following table (Table 4.12) shows the percentage of leucine in dietary protein, the 24 hour urinary N excretion and the equivalent calculated leucine oxidation rate over 24 hours (E_U) and compares this value with the oxidation rate derived from flux and expired ¹⁴CO₂ (E_L).

TABLE 4.12. 24-Hour Leucine oxidation rates derived from urinary N excretion (E_U) and from flux and expired $^{14}\text{CO}_2$ measurements (E_L). The comparison of the two oxidation rates suggests the underestimate of the flux due to internal recycling.

GROUP & NAME	% Leucine in Dietary Protein	24 hr Urinary Excretion		E_L	$E_L/E_U \times 100 \%$
		g Protein (N \times 6.25)	mmol Leu (E_U)	mmol Leu/24 hrs	
<u>OBESE DIURNAL</u>					
FB	9.80 %	89.7	65.8	45.4	69.0 %
SH	9.77 %	73.3	54.4	39.2	72.1 %
DH	9.77 %	84.5	61.0	37.5	61.5 %
MH	9.79 %	85.4	61.8	49.6	80.3 %
ML	9.21 %	-	-	(34.8)	-
MT	9.81 %	85.2	62.8	42.6	67.8 %
MW	9.51 %	85.3	60.3	36.3	60.2 %
TD	9.43 %	88.9	61.5	37.9	61.6 %
EK	10.09 %	71.9	54.7	40.3	73.7 %
RO	9.71 %	74.3	54.7	34.6	63.3 %
<u>OBESE</u>					
MEAN \pm S.D.	9.69 %	82.1	59.7	40.4	67.7 %
	± 0.24	± 6.9	± 4.1	± 4.8	± 6.7
<u>CONTROL</u>					
MG	9.76 %	67.3	49.8	41.2	83.2 %
JS	9.76 %	84.6	60.4	44.9	74.3 %
JCW	9.24 %	92.9	62.8	29.2	46.5 %
RWS	9.51 %	101.3	70.1	37.0	52.8 %
JKG	9.46 %	76.0	53.8	36.8	68.4 %
<u>CONTROL</u>					
MEAN \pm S.D.	9.55 %	84.4	59.4	37.8	65.0 %
	± 2.2	± 13.4	± 7.9	± 5.9	± 15.1

These data show that for both obese and non-obese subjects, over a 24 hour period which includes both feeding and fasting phases, 24 hour whole body protein synthesis, oxidation, and flux, are all underestimated by some 33%.

There are 2 factors that are likely to account for the discrepancy between this figure and the value of 20% found in patient E.E. (see previous section) and Golden & Waterlow (1977). Firstly, it might well be expected that during fasting, the amount of amino acid liberated by protein breakdown and then reutilised for synthesis without mixing in the plasma pool, should increase, especially since the earlier data suggest there is an increase in breakdown during the fasting phase. Secondly, both E.E. and Golden & Waterlow's subjects were receiving their protein intake over 24 hours, and therefore at a much slower *hourly* rate of protein intake than in these subjects with a 12 hour feeding phase. Consequently the flux rates of both E.E. and Golden & Waterlow's subjects are much lower than the 12 hour feeding phase values in the above obese and control subjects. Different flux rates may well be associated with different rates of internal recycling.

The second approach to estimating this error in plasma measurements of flux, is to measure excretion of urinary N during both feeding and fasting phases, and correct these 12 hour excretion rates for the changes in body urea pool size that occur with feeding and fasting. These changes are estimated as a product of measured plasma urea-N values and total body water. Then again assuming that the proportion of urinary N derived from leucine is the same proportion as leucine in dietary protein until N-balance is achieved, and thence as in whole-body protein, leucine oxidation can be calculated from the corrected urinary N excretion values from both 12 hour phases, and compared with the flux-based estimates of oxidation. Total body water is computed from the following

formula from Watson *et al* (1980).

$$TBW = 20.03 - (0.1183 \times \text{Age in yrs}) + (0.3626 \times \text{Wt in Kg}).$$

TABLE 4.13. Estimation of leucine oxidation from urinary-N excretion, over both feeding and fasting 12-hour phases, by correcting the 12-hour urinary-N excretion for changes in body urea pool size.

(Control subject JKG).

	CHANGE IN PLASMA UREA-N mg/l	CHANGE IN WHOLE-BODY UREA-N g N	URINARY-N EXCRETION	
			AS MEASURED g N/12 hrs	CORRECTED g N/12 hrs
FEEDING 12 HOURS	+ 24.3	+ 0.952	7.03	7.98
FASTING 12 HOURS	+ 24.3	+ 0.952	5.13	4.18
TOTAL 24 HOURS	-	-	12.16	12.16

PHASE	% LEUCINE IN DIETARY PROTEIN	CORRECTED URINARY EXCRETION		E_L mmol Leu	$E_L/E_U \times 100 \%$ (%)
		g Protein (N x 6.25)	mmol Leu (E_U)		
FEEDING 12 Hrs	9.46 % in 66.7 g Protein	49.9	36.0	24.9	69.2 %
FASTING 12 Hrs	-	26.1	17.8	12.0	67.5 %
TOTAL 24 Hrs		76.0	53.8	36.9	68.6 %

From these data there seems to be little change detectable, as a result of feeding and fasting, in the relationship between oxidation rates derived from N excretion and those derived from flux and expired $^{14}\text{CO}_2$. However there may still be some insensitivity to acute turnover changes in the urinary nitrogen method, even with urea space corrections applied, because expansion in the urea pool still lags a few hours behind protein intake. Nevertheless, in both obese and non-obese subjects, the (1- ^{14}C)leucine infusion method appears to underestimate true precursor pool flux, and thus protein synthesis, and oxidation rates, by some 33%, at least over 24 hours when the daily intake of 70 g protein is administered over a 12 hour feeding period.

There is another important factor that requires appreciation before changes in body protein mass are considered. Despite a protein intake of about 70 g protein per day and an energy intake that was just above requirement in most subjects (energy balances are shown in Part B), all subjects were in significant negative N-balance*, and consequently negative leucine balance. The following table (Table 4.14) shows the mean nitrogen and leucine balances for the obese and control groups. As in the previous tables, it is assumed that up until N-balance, leucine is excreted in the same proportion to protein as in protein intake (i.e. approx. 9.7%), but for the N excreted in excess of intake, the proportion of leucine in protein excreted is 8%, as in 'whole body protein'. The individual intakes, excretion, and balances for both groups are shown in Appendix 2.7.

* N-balances here represent the balance between N intake and urinary N excretion, and are thus approximations for true N-balance. Faecal, integumental, or other losses have not been measured. However use of standard digestibility factors (Watt & Merrill, 1963) partly account for faecal N loss.

TABLE 4.14. 24-Hour mean (\pm S.D.) protein and leucine balances for 10 obese and 5 control subjects. (Excreted 'Protein' is $N \times 6.25$.)

GROUP	INTAKE		EXCRETION (urine)		BALANCE	
	Protein g/d	Leucine mmol/d	Protein g/d	Leucine mmol/d	Protein g/d	Leucine mmol/d
OBESE	72.6	53.6	82.1	59.7	-9.6 ± 6.8	-5.8 ± 4.2
CONTROL	67.2	48.8	84.4	59.4	-17.2 ± 11.7	-10.5 ± 7.1

All patients, both obese and control, were in quite marked negative nitrogen balance (-1.5 gN/d for obese; -2.8 gN/d for Controls) despite the 'Normal' dietary intake. This interesting phenomenon is also seen in many other patients (not listed here), and the typical N excretion pattern is one of increasing negative balance over the first 3-4 days of admission to hospital, even though an adequate energy/high protein diet is consumed. It seems more likely that this effect is related to immobilisation (particularly as required for ($1-^{14}\text{C}$)leucine infusions). This likelihood is further reinforced by the achievement of complete N-balance in 6 colleagues participating in (^{15}N)glycine studies (Garlick *et al.*, 1980), but who were active and working as usual during the daytime. In complete contrast, our Control group (which also consisted of colleagues) were significantly and suddenly restricted in their movements for the 24 hours of the ($1-^{14}\text{C}$)leucine infusion.

From the discussion above, it is apparent that changes in amino acid pool size are very small and will scarcely affect the size of any diurnal changes in protein synthesis; the correction factor for

underestimate of flux by the (1-¹⁴C)leucine method will however have a large effect on results; and the fact that all the subjects were in negative nitrogen balance needs to be taken into account. Bearing in mind that the following corrections for underestimate of flux assume leucine oxidation and protein synthesis share the same precursor pool, the following tables show the resultant corrected mean values of protein turnover over 24 hours in obese patients (Table 4.15) and Control subjects (Table 4.16).

TABLE 4.15. Mean 24-hour protein turnover measurements in 10 obese patients on the diphasic 'Normal' Diet regimen, corrected for a 33% underestimate of flux in both feeding and fasting phases. Expressed in millimoles of leucine and *g of Protein*.

PHASE	Q	Z	B	E*	I
	millimoles of Leucine per 12 Hours <i>g Protein per 12 Hours</i>				
FEEDING	151.2	108.7	97.6	42.5	53.6
12 HOURS	247.9	178.3	160.0	57.5	72.6
FASTING	96.7	79.7	96.7	17.0	0
12 HOURS	158.6	130.7	158.6	'24.7'	0
TOTAL	247.9	188.4	194.3	59.5	53.6
24 HOURS	406.5	308.9	318.6	'82.2'	72.6

* E expressed in *g Protein*, is 'Intake Protein' as far as protein balance, and thereafter is 'Whole Body Protein'. Thus 24 hour protein oxidation (82.2 g) is a combination of the 2 types of protein.

If these corrections are valid, then the data in Table 4.15 would indicate the following features of 24 hour protein turnover, some in marked contrast to the previous 'uncorrected' findings.

- (1) Diurnal feeding/fasting changes in protein turnover now appear somewhat different:
 - Protein synthesis still drops by 27%;
 - Protein oxidation still drops by 60%;
 - But protein breakdown remains completely unchanged.
- (2) In the 12-hour feeding phase, although in terms of leucine the discrepancy between Z and B equals that between E and I, in terms of protein this is not the case since the leucine concentration in food protein and whole body protein differ. The difference between E and I indicates that only 20% of protein intake is stored, 80% being oxidized during the 12 hour feeding period. The leucine that is retained is then reflected in the discrepancy between Z and B, and this indicates an increase in whole body protein-bound leucine of 11.1 millimoles, equivalent to an increase in body protein mass of 18.2 g of protein during the feeding 12 hours.
- (3) During the fasting 12 hours, the discrepancy between Z and B indicate a net loss of protein bound leucine of 17 millimoles of leucine, equivalent to a decrease in whole body protein mass of 27.9 g protein during this phase.
- (4) Thus over the whole 24 hour period, in these obese patients there is a net loss of 5.9 millimoles of leucine from the body. In terms of net loss from the body's protein mass (Z - B), this is equivalent to a loss of 9.7 g Protein.

With this same correction for a 33% underestimate of flux applied to the Control group results, the absolute values and proportional changes are almost identical to the corrected values from the obese group. Table 4.16 shows this, and again demonstrates the diurnal feeding/fasting changes, with a 27% drop in whole body synthesis, a 56% drop in oxidation rate, and no change in protein breakdown rate.

TABLE 4.16. Mean 24-hour protein turnover rates for the Control Group, corrected for a 33% underestimate of flux. Expressed in millimoles of leucine per 12 hours, and *g Protein per 12 hours*.

PHASE	Q	Z	B	E [*]	I
	millimoles of Leucine per 12 Hours g Protein per 12 Hours				
FEEDING	141.3	102.1	92.5	39.2	48.8
12 HOURS	231.7	167.4	151.7	53.8	67.2
FASTING	91.5	74.3	91.5	17.2	0
12 HOURS	150.0	121.8	150.0	'25.6'	0
TOTAL	232.8	176.4	184.0	56.4	48.8
24 HOURS	381.7	289.3	301.7	'79.4'	67.2

* E as g Protein is 'Intake Protein' (leucine = 9.55%) until protein balance is achieved and thereafter is 'Whole Body Protein' (leucine = 8.0%).

With these corrections applied, the protein turnover data for both obese and Control group subjects in Tables 4.15 and 4.16 are now in agreement with measured nitrogen balance data. Yet even so, the validity of these corrections depends on the assumption that

oxidation and synthesis share the same precursor pool throughout the body, and this assumption may introduce significant error, since leucine is thought to be largely oxidised in muscle (Young, 1970; Goldberg & Chang, 1978; Elia et al, 1980), whilst muscle only contributes about half of total whole-body protein synthesis (Waterlow, Garlick & Millward, 1978).

Nevertheless, irrespective of any corrections, the results clearly show a 27% drop in whole body protein synthesis, and a 60% drop in oxidation, in the change from a feeding to a fasting phase. Whether this change also causes a 35% increase in whole body breakdown, as initially shown, and whether 50% of protein intake is stored during the feeding phase, is much more dubious. If the (1-¹⁴C)leucine infusion method does underestimate not only oxidation, but also flux, by some 33% (under the dietary conditions of this study), then it would appear that protein breakdown has no diurnal change, and only 20% of dietary protein is stored during the feeding phase.

Despite this uncertainty about the magnitude of these breakdown changes, it has been established in this Section that considerable diurnal changes in protein turnover do occur, and are characterised by an acute responsiveness to changes in the level of protein intake. Protein turnover rates and their diurnal changes appear to be identical in obese and non-obese subjects at least on normal dietary intakes, and there seems to be no evidence of natural circadian rhythms contributing to the diurnal changes. This section has also shown that although fluctuations do occur in the body's free amino acid pools, these are comparatively very small changes when compared with the changes in whole-body protein synthesis.

However the (1-¹⁴C)leucine method may significantly underestimate rates of protein oxidation and flux by some 33%, in both obese and non-obese subjects, and it has been suggested that the level of this error may be related to the rate of protein intake.

It was originally thought that such a remarkable decrease as a 38% fall in protein synthesis, resulting from 3 weeks of protein-free low energy diet, must be a slow adaptive change, and it was planned to investigate the time-scale of this change with repeated (¹⁵N)glycine measurements (Section 5). However, this Section has shown that over $\frac{2}{3}$ of that drop in synthesis occurs routinely in obese and non-obese adults, within a few hours of post-absorptive fasting. The (¹⁵N)glycine investigations in the next section are left to cope with the remaining small decrease.

SECTION 5 (1-¹⁴C)LEUCINE vs (¹⁵N)GLYCINE

RESULTS 3

5.1. (1-¹⁴C)LEUCINE COMPARED WITH (¹⁵N)GLYCINE

5.1.1. Continuous intravenous (¹⁵N)glycine

5.1.2. Multiple oral dose (¹⁵N)glycine

5.1.3. Single (oral and I.V.) dose

(¹⁵N)glycine

5.2. MONITORING PROTEIN TURNOVER WITH (¹⁵N)GLYCINE

RESULTS 3

This section presents protein turnover measurements that were made by methods other than (1-¹⁴C)leucine. As outlined in previous sections, once it was found that a prolonged protein-free diet caused major changes in whole body protein turnover, it was decided to use (¹⁵N)glycine to attempt to monitor the rate of fall in protein synthesis. The use of the stable isotope ¹⁵N allowed frequent, repeated measurements of protein turnover to be made, this being impossible with radioactive tracers such as (1-¹⁴C)leucine, because radiation hazard strictly limits their cumulative dosage. However (¹⁵N)glycine measurements were also made in a number of subjects simultaneously with (1-¹⁴C)leucine infusions, and thereby offered comparative measurements of whole body protein turnover, valuable to the understanding of both methods.

It was most fortuitous that these studies coincided with the programme of Professor J.C. Waterlow to develop an ammonia end-product method using (¹⁵N)glycine. Over the last decade the most commonly used method of measuring whole body protein turnover has been that adopted by Picou & Taylor-Roberts (1969) in which (¹⁵N)glycine is administered continuously by intravenous or oral routes, until the abundance of ¹⁵N in urinary urea reaches a plateau. The disadvantage of this method is that in adults it takes 2 or 3 days for urea to reach this plateau, and sometimes even longer (eg. Steffes et al, 1976), particularly in subjects with a low rate of urea excretion and turnover.

However Waterlow et al (1978) suggested the use of ammonia as the urinary end product, since the abundance of ¹⁵N rises to a plateau in about 9 - 12 hours (see Fig. 2.5), a great advantage over the urea

end-product method. Some of the testing of this method was done by comparing its estimates of whole body flux and synthesis with the results from (1-¹⁴C)leucine infusions, in both obese and the Control group of subjects. Further modifications of this method were also introduced and tested, including the administration of (¹⁵N)glycine by single dose, either intravenously or orally. These developments were also tested in the obese subjects, and against results obtained from simultaneous measurement with (1-¹⁴C)leucine.

These (¹⁵N)glycine measurements were thus part of a collaborative programme, in which the (¹⁵N)glycine method was used to obtain protein turnover measurements as an independent comparison with the (1-¹⁴C)leucine results, the changes in whole body protein synthesis were monitored during prolonged low protein dieting, measurements were made in a number of other conditions such as total fasting, and infected babies, and through all these studies the underlying aim of the programme was to test the limits, extend, and validate the NH₃ end product method.

In view of the collaborative nature of much of this work, this section mainly presents relevant (¹⁵N)glycine results, with little in-depth discussion about the development of the NH₃ end-product method. The results are shown in two sections here, the first showing the agreement reached between (¹⁵N)glycine and (1-¹⁴C)leucine in the estimation of protein turnover, and the second section showing attempts to monitor the progressive changes in protein turnover with prolonged low protein diets. The principles involved in the use of this end-product method, and the practical details of the method, have been outlined in Sections 1 and 2.

5.1. A COMPARISON OF (1-¹⁴C)LEUCINE AND (¹⁵N)GLYCINE/NH₃ END-PRODUCT METHODS IN ESTIMATING PROTEIN TURNOVER

Golden & Waterlow (1977) made simultaneous measurements of protein turnover by continuous administration of both (1-¹⁴C)leucine and (¹⁵N)glycine with NH₃ end product, and found that under their continuous feeding conditions, the NH₃-end product method appeared to give slightly (but consistently) lower rates of protein synthesis. The preliminary results of more extensive comparisons between the (1-¹⁴C)leucine method and (¹⁵N)glycine (both single dose and multiple dose) have been given by our group (Garlick, Clugston & Waterlow, 1980), and these showed that although absolute values from the two methods were not identical, the same proportional changes in protein turnover as a result of low protein diets were measured.

5.1.1. Continuous intravenous infusion of (¹⁵N)glycine

The first set of results is for the Control group of non-obese subjects, 4 of whom had simultaneous 24 hour infusions of (1-¹⁴C)leucine and (¹⁵N)glycine. Figure 2.5 (Section 2) has already clearly shown the rise of the abundance of ¹⁵N in urinary NH₃ to a plateau, changing again once the feeding 12-hour phase is completed and achieves a fasting phase plateau. These plateau values are used in the same way as plasma leucine specific activity to calculate flux Q. Table 5.1 sets out the mean values for the group, but the individual values for each subject are tabulated in Appendix 3.1.

TABLE 5.1. Mean (\pm S.D.) protein turnover rates in 4 non-obese 'Control' Group subjects, measured by simultaneous intravenous infusion of (^{15}N)glycine and (1- ^{14}C)leucine, (1- ^{14}C)leucine results in italics), over 24 hours.

PHASE		Q	Z	B	E
		g Protein per 12 Hours			
FEEDING 12 HOURS	(Gly)	134.9 \pm 24.7	90.8 \pm 26	68.3 \pm 28	44.1 \pm 8.5
	(Leu)	156.3 \pm 20	112.7 \pm 28	75.8 \pm 21	43.6 \pm 8.5
FASTING 12 HOURS	(Gly)	75.6 \pm 17	31.5 \pm 11	75.6 \pm 17	44.1 \pm 8.6
	(Leu)	101.7 \pm 17	82.9 \pm 17	101.7 \pm 17	18.8 \pm 4
TOTAL 24 HOURS	(Gly)	210.5 \pm 38	122.3 \pm 35	143.9 \pm 40	88.2 \pm 17
	(Leu)	258 \pm 36	190.7 \pm 52	177.5 \pm 36	62.4 \pm 11

Statistically (by matched pair t-test), the results of (^{15}N)glycine and (1- ^{14}C)leucine are in much better agreement in the feeding phase (N.S.D. between the methods for Q, Z, B, E), than in the fasting phase ($p < .05$ for all Q, Z, B, E) or for the total 24 hour period. However in all phases this (^{15}N)glycine method appears to give consistently lower flux results than the (^{14}C)leucine method (as was similarly found by Golden & Waterlow, 1977).

A difficulty highlighted here is the use of urinary N excretion to represent protein oxidation rates for intervals of less than 12 hours. In Table 5.1 the 24 hour N-excretion, representing 88.2 g Protein, was halved for the 12 hour rates. In fact the 12 hour urinary-N excretion profiles, for these 4 non-obese subjects, show that they excreted some 55.7% of their 24 hour urinary-N output during their feeding 12 hours, and the remaining 44.3% during the fasting 12 hours, as shown in Table 5.2.

TABLE 5.2. Urinary nitrogen excretion over Feeding and Fasting 12-hour periods, for 4 non-obese (2 males, 2 females) subjects of the 'Control' Group. (Protein = N x 6.25)

NAME	FEEDING 12 HOURS		FASTING 12 HOURS		TOTAL 24 HOURS	
	g Protein	% of 24 Hr excretion	g Protein	% of 24 Hr excretion	g Protein	% of 24 Hr excretion
MG	35.8	53.2 %	31.5	46.8 %	67.3	100 %
JS	50.9	60.2 %	33.7	39.9 %	84.5	100 %
JCW	56.1	60.4 %	36.8	39.6 %	92.9	100 %
RWS	49.4	48.7 %	51.9	51.2 %	101.3	100 %
MEAN		55.6 %		44.3 %		
± S.D		±5.7	.	±5.6		

However, in Section 4 it has been shown with both leucine oxidation and correction of urinary-N for changes in whole body urea space, that there is usually a 60% drop in protein oxidation between feeding and fasting phases, so that about 70% of protein oxidation occurs during the feeding 12 hours, and the remaining 30% during the fasting 12 hours.

If this proportional oxidation pattern were applied to the urinary-N based oxidation rates in Table 5.1, they would probably be a reasonable approximation of genuine oxidation - but then the (^{15}N)glycine's estimation of synthesis during the feeding period would become even lower (73.2 g Protein per 12 Hours) whilst in the fasting period the estimate of synthesis would improve (49.1 g Protein per 12 Hours) but still remain substantially below the value estimated by ($1\text{-}^{14}\text{C}$)leucine. However with these corrections, the magnitude of the diurnal drop in protein synthesis (33%) now becomes much closer to that shown by ($1\text{-}^{14}\text{C}$)leucine.

It does, however, appear that both methods significantly underestimate flux (and hence synthesis and breakdown). In the previous section (Section 4) it was suggested that the (1-¹⁴C)leucine method underestimates flux by some 33%. If this is the case, then the intravenous (¹⁵N)glycine method, using urinary ammonia as end product, may be underestimating true flux by some 45%.

The consistent relationship between the results from the two methods (in which (¹⁵N)glycine gives consistently lower estimates of flux and synthesis than the (1-¹⁴C)leucine method) is maintained even through prolonged dietary changes. The following figure (Figure 5.1) shows this relationship for whole body protein synthesis in one of the obese patients (R.O).

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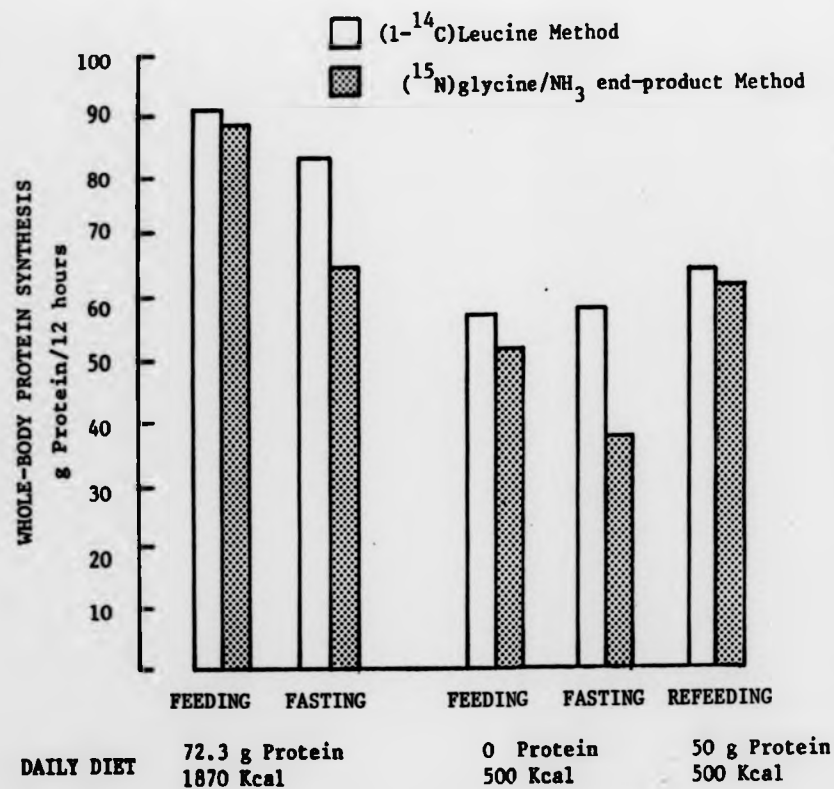


FIGURE 5.1. Whole body protein synthesis in an obese patient (R.O.) measured simultaneously by (1-¹⁴C)leucine infusion and (¹⁵N)glycine intravenous infusion. The intravenous (¹⁵N)glycine method seems to give persistently lower results, particularly in Fasting periods.

5.1.2. Multiple (hourly) oral doses of (^{15}N)Glycine

Protein turnover measurements using this method were only made during daytime feeding periods, but the method was used to obtain results for patients on Diet P and Diet O for 3 weeks, as well as initially on the 'Normal' Diet. The results have already been presented in Table 3.11 (Section 3), and contrary to the intravenous (^{15}N)glycine results, the multiple oral doses method appears to produce higher absolute values for flux and synthesis, than the ($1\text{-}^{14}\text{C}$)leucine method. However, if the ($1\text{-}^{14}\text{C}$)leucine results were to be corrected for their possible 33% underestimate of flux and synthesis, they would be significantly higher than the (^{15}N)glycine multiple dose measurements.

Despite these differences in absolute values, it was shown (Table 3.12) that the proportional changes in protein turnover, resulting from 3 weeks on Diet P and Diet O, were almost identical, measured by multiple doses of (^{15}N)glycine and by ($1\text{-}^{14}\text{C}$)leucine infusion.

5.1.3. Single dose of (^{15}N)Glycine (oral and intravenous)

It was hoped that with the much greater convenience that the single dose technique offered, protein turnover measurements would still compare reasonably well with ($1\text{-}^{14}\text{C}$)leucine measurements. As mentioned in Sections 1 and 2, the use of the single dose method introduced its own problems, such as determining the appropriate collection period when sufficient isotope had cleared the metabolic pool but the contribution from recycling of ^{15}N was still minimal, and the necessity to collect serial urine samples because of variation in the rate of urinary NH_3 excretion.

Nevertheless, by taking 3-hourly or 6-hourly serial urine collections and choosing 12 hours (in later studies this was changed to 9 hours) as an appropriate collection time for adequate clearance of the isotope, measurements of whole body protein turnover were made by this method on a group of obese patients, some of whom had simultaneous (1-¹⁴C)leucine infusions, on the 'Normal' Diet regimen, and then after 3 weeks on Diet O. The measurements were made only during feeding periods, and are thus only comparable with feeding phase (1-¹⁴C)leucine values. In the table below (Table 5.3), only 2 of the 6 patients in the 'Normal' Diet group had simultaneous (1-¹⁴C)leucine infusions, so the leucine measurements are the standard 'Normal' Diet values from Table 3.7. The 3 patients in the 'Diet O' group all had simultaneous (1-¹⁴C)leucine infusions, so the values given for both measurement methods are specifically theirs. Appendix 3.2 contains the individual (¹⁵N)glycine measurements for each patient, and Table 5.3 shows the mean (\pm S.D.) for each group.

TABLE 5.3. Comparison of oral single dose (¹⁵N)glycine/NH₃ end-product measurement of protein turnover with (1-¹⁴C)leucine (*italics*). The results show Mean (\pm S.D.) for 6 obese patients on 'Normal' Diet, and 3 obese patients after 3 weeks on Diet O. (Feeding 12 Hours only.)

FEEDING PHASE		g Protein per 12 Hours			
		Q	Z	B	E
NORMAL DIET	(¹⁵ N)gly (n=6)	169.3 \pm 61	131.9 \pm 62	98.3 \pm 62	37.4 \pm 3
	(¹⁴ C)Leu (n=19)	167.5 \pm 17	122.9 \pm 17	83.0 \pm 17	44.6 \pm 8
DIET O (n=3)	(¹⁵ N)gly	70.6 \pm 4	60.8 \pm 6	70.6 \pm 4	9.8 \pm 2
	(¹⁴ C)Leu	87.9 \pm 15	80.5 \pm 14	87.9 \pm 15	7.4 \pm 2

These data show a remarkably good comparison between the two measurement methods. In fact synthesis (Z) values would be even more closely matched if oxidation rate values (E) based on N-excretion, were corrected for changes in urea space, as shown in previous section (Section 4). Statistical testing (t-test) shows no significant difference between the 2 methods for all the protein turnover parameters.

Thus the single oral dose technique with (^{15}N)glycine, gives very similar (mean) estimates of protein turnover, to the ($1\text{-}^{14}\text{C}$)leucine method, although it appears to produce a much wider scatter of results at least on the 'Normal' Diet. Consequently if the ($1\text{-}^{14}\text{C}$)leucine method really does underestimate true flux by 33%, then it appears that this particular version of the (^{15}N)glycine method does also.

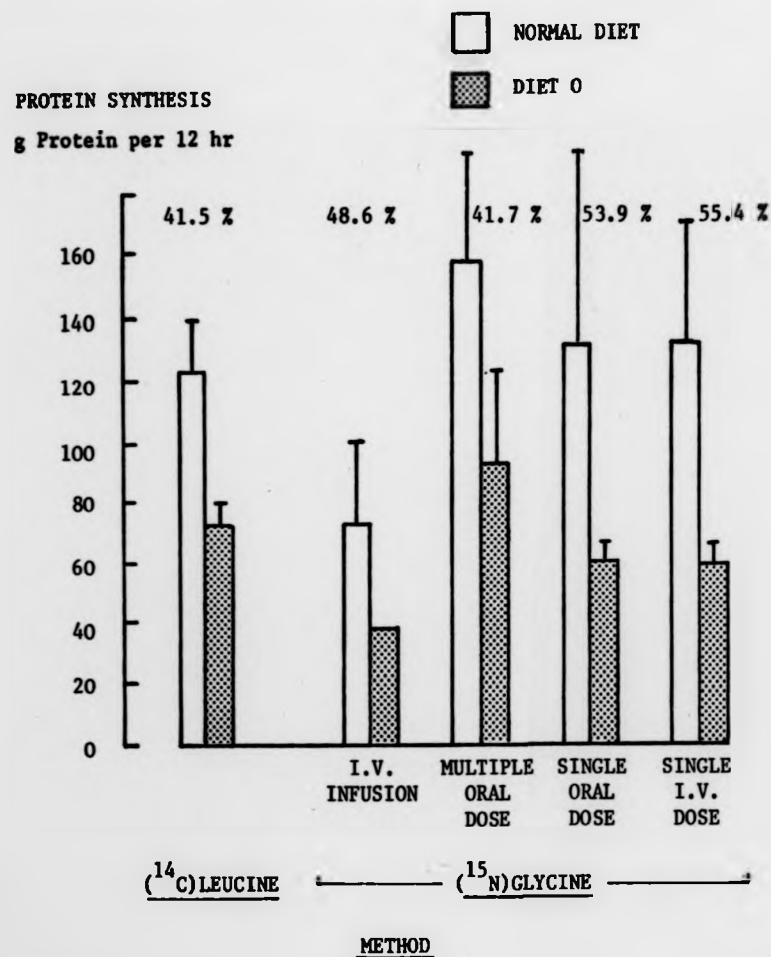
The single intravenous dose method with (^{15}N)glycine was also repeatedly tried and tested in both obese and normal subjects, and showed a similar close relationship to ($1\text{-}^{14}\text{C}$)leucine values for protein turnover, as the single dose oral (^{15}N)glycine method. A group of 4 normal, slim young adults ('Starvation' Group, see 5.2) and 2 older non-obese adults, were measured by the single intravenous dose method of (^{15}N)glycine, and the values in Table 5.4 clearly show that the (^{15}N)glycine method, despite quite wide scatter results, give mean values that compare well with those of ($1\text{-}^{14}\text{C}$)leucine.

TABLE 5.4. The intravenous single dose (^{15}N)glycine/ NH_3 end-product method, used to measure protein synthesis and oxidation in 5 normal adults during their feeding 12-hours on a 'Normal' Diet. Mean (\pm S.D.) values for measurements made with (1- ^{14}C)leucine in another 6 normal adults (2 of whom are included in the (^{15}N)glycine group) are supplied for comparison (*italics*).

NAME	PROTEIN SYNTHESIS Z g Protein per 12 Hours	PROTEIN OXIDATION E
RM	146.7	52.8
MB	112.1	25.2
IP	175.4	56.1
PM	64.3	49.4
JKG	126.3	37.7
VT	165.0	46.2
MEAN	131.6	44.6
\pm S.D.	± 40	± 11
<u>(^{14}C)Leucine Group</u>		
MEAN	110.3	41.2
\pm S.D.	± 22	± 8

When these and other measurements done by both (1- ^{14}C)leucine and the different (^{15}N)glycine methods are compared, it is apparent that although the different (^{15}N)glycine methods are all characterised by a rather wide scatter of results, the magnitude of change in protein turnover as a consequence of prolonged protein-free dieting, is very similar by all methods including (1- ^{14}C)leucine. This can be seen in Figure 5.2.

FIGURE 5.2. Mean (\pm S.D.) whole body protein synthesis rates, measured by $(1-^{14}\text{C})$ leucine and 4 different methods of (^{15}N) glycine, on 'Normal' Diet and after 3 weeks on Diet O. The percentage changes from Normal diet to Diet O are shown for each method.



A major advantage of the single dose (^{15}N)glycine method is its potential for frequent repeatability in the same subject, largely because of the rapid rise to plateau of the cumulative excretion of ^{15}N in urinary ammonia. This capability was tested in patient E.E. who was having continuous hourly feeding for 24 hours and a simultaneous ($1\text{-}^{14}\text{C}$)leucine infusion (see Section 4, Table 4.8). Her first (^{15}N)glycine (I.V. single dose) measurement of protein turnover was completed over the usual 9 hour collection period, and then after an interval of only 9 hours, a second I.V. single dose (^{15}N)glycine measurement of protein turnover was made. Figure 5.3 shows the change in ^{15}N abundance in urinary NH_3 over the 27 hour period, and Table 5.5 below shows the excellent agreement of the two (^{15}N)glycine tests with each other, and with ($1\text{-}^{14}\text{C}$)leucine.

Atom % Excess
($\times 1000$)

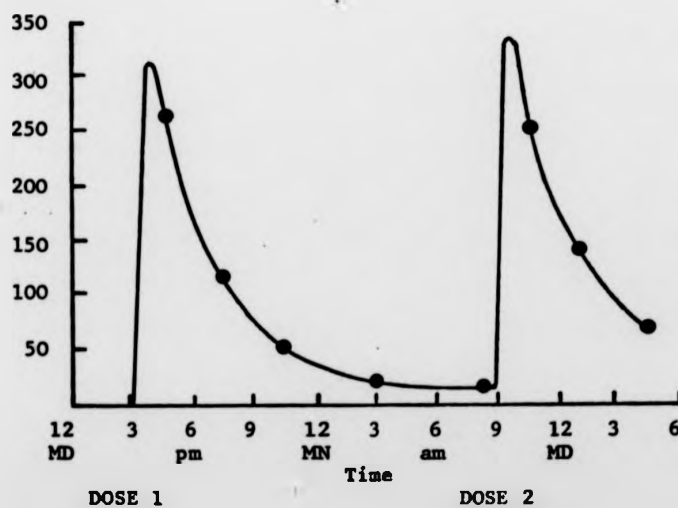


FIGURE 5.3. Abundance of ^{15}N in Urinary NH_3 . Two measurements of protein turnover by single dose I.V. (^{15}N)glycine, the second test commencing only 9 hours after completion of the first. (Patient E.E.)

TABLE 5.5. Protein turnover in a continuously fed obese patient (E.E) measured by single dose I.V. (^{15}N)glycine on 2 occasions only 9 hours apart, and also continuously with ($1\text{-}^{14}\text{C}$)leucine (*italics*).

	Q	Z	B	E
	g Protein per 12 Hours			
<u>1st 9 Hours</u>				
(^{15}N)glycine	93.5	67.4	57.4	26.0
($1\text{-}^{14}\text{C}$)leucine	103.6	79.4	59.6	24.2
2nd 9 Hours = Interval				
<u>3rd 9 Hours</u>				
(^{15}N)glycine	95.4	69.4	59.3	26.0
($1\text{-}^{14}\text{C}$)leucine	101.6	76.1	57.6	25.5

The reasonable similarity between the single dose (^{15}N)glycine values and the ($1\text{-}^{14}\text{C}$)leucine values for protein turnover, and the advantages of convenience, safety and ability to be used repeatedly, encouraged us to use the single dose (^{15}N)glycine method to monitor the rate of change in protein turnover in a number of clinical situations. It was initially tried in the obese patients on protein-free or low protein diets, and as this proved successful, it has subsequently been used in other studies such as a 'starvation' study (see 5.2), monitoring the recovery of babies from meningitis (to be written-up), and a study investigating the effect of typhoid/cholera vaccination on protein turnover (Garlick *et al*, 1980).

5.2. MONITORING PROTEIN TURNOVER CHANGES WITH SINGLE DOSE (^{15}N)GLYCINE/ NH_3
END-PRODUCT METHOD.

Having shown that the single dose (^{15}N)glycine method could measure protein turnover daily if necessary, and that the results were reasonably comparable with ($1\text{-}^{14}\text{C}$)leucine measurements, particularly in terms of proportional changes, a number of obese patients were measured on frequent occasions by this method during their weight reduction courses on Diet P and Diet O. In addition, for some obese patients protein intake was changed to different levels, and the effect of this on protein turnover measured.

It has been shown in Section 3 that after 3 weeks on 500 Kcal (2.1 MJ) and with complete absence of protein, that whole body protein synthesis drops by some 40%. It has further been shown in Section 4 that the absence of protein intake for only a few hours (as in normal overnight post-absorptive fasting) results in an acute drop of 27% in the rate of protein synthesis which remains at this depressed level until protein intake is recommenced at the end of the fasting 12 hours. It therefore appears that the remaining 13% drop in synthesis must occur at some stage over the 3 weeks of protein-free diet, and it was hoped that frequent measurements with (^{15}N)glycine would elucidate the time-course of this decrease.

FIGURE 5.4. Whole body protein synthesis rates in 4 obese patients on Diet O (500 Kcal/O Protein) for 21 days, measured by oral single dose (^{15}N)glycine/ NH_3 end-product method. The initial (Day 0) measurement was made during feeding phase of 'Normal' Diet.

PROTEIN SYNTHESIS

g Protein per 12 Hours

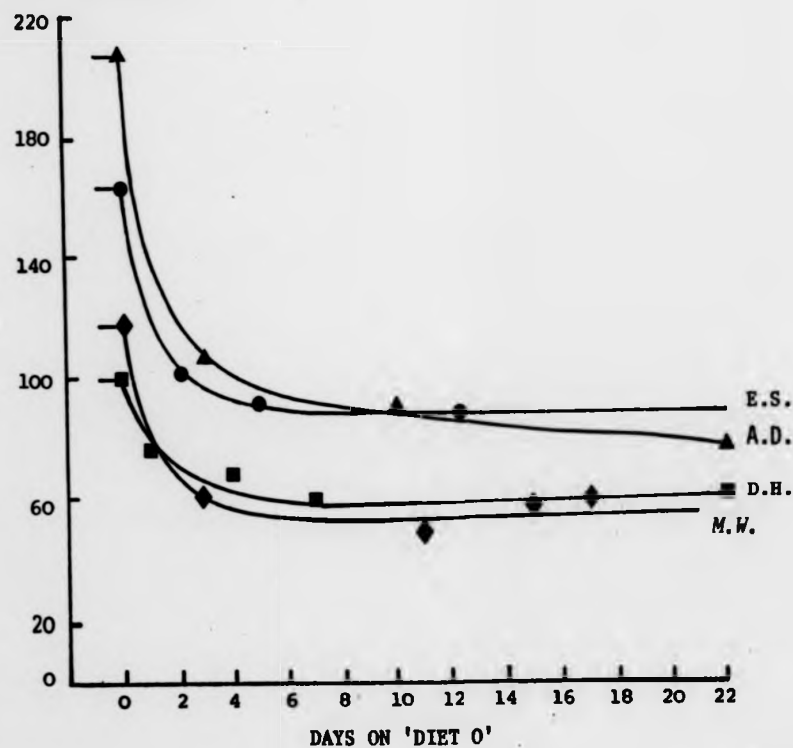


Figure 5.4 shows this time course for 4 obese patients, and although absolute values are spread quite widely, the relative rate of fall in protein synthesis is very similar for each patient. The total fall in protein synthesis for the group as a whole is approximately 48%. However protein synthesis rates have already dropped 40% by Day 2, 46% by Day 4, and 48% by Day 6 after which there is no significant further fall.

A similarly rapid fall in whole body protein synthesis is seen in starvation. When 3 young healthy adults embarked on a one week starvation programme, consuming only mineral water, their whole body protein turnover was measured on three occasions during the week by the single dose intravenous (^{15}N)glycine method. Within 2 days of complete fasting, protein synthesis rates had fallen by some 50% and remained at that level for the rest of the week. Their oxidation rates appeared to fall more slowly but this delay was partly a result of slow excretion from the urea pool. Table 5.6 shows the results for protein synthesis and oxidation.

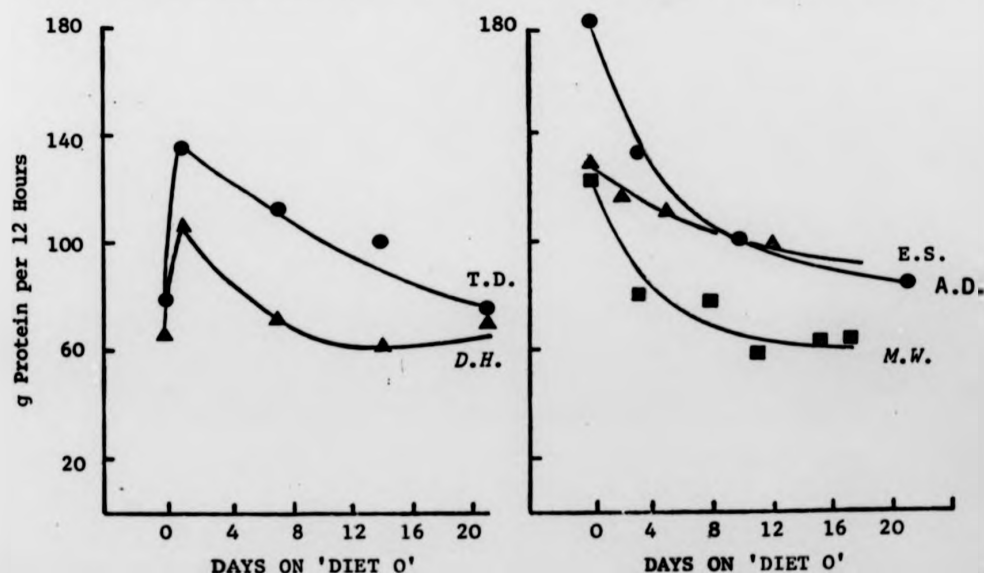
TABLE 5.6. Mean (\pm S.D.) whole body protein synthesis and oxidation rates for 3 young adults undergoing 1 week of starvation.

	SYNTHESIS Z g Protein per 12 Hours	OXIDATION E
NORMAL DIET	144.7 ± 32	44.7 ± 17
DAY 2 STARVATION	62.6 ± 6	35.6 ± 16
DAY 5 STARVATION	78.1 ± 11	32.6 ± 7

There is more difficulty with protein breakdown however, because the interpretation of its changes appears to depend much more on the absolute values obtained for flux - and these vary widely with (^{15}N)glycine methods. Figure 5.5 indicates this dilemma, showing that if the drop in flux that occurs when changing from 'Normal' Diet to the protein-free diet, is a small one (ie. smaller than the drop in protein intake), then protein breakdown will initially *increase* (Fig. 5.5A) before gradually decreasing with the subsiding flux. If the absolute drop in flux with the change in diets is a large one (ie. larger than the drop in protein intake) then calculated protein breakdown will simply decrease from Day 0 (Fig. 5.5B). The

FIGURE 5.5. Protein Breakdown, measured by (^{15}N)glycine. The pattern of change in protein breakdown rates particularly depends on the magnitude of the drop in flux.

(A) When Drop in flux < Drop in Intake (B) When Drop in flux > Drop in Intake



(1-¹⁴C)leucine data, however suggests an initial increase in breakdown is the likely pattern. In Section 4 (Results 2) it was shown from (¹⁴C)leucine data that there was a diurnal increase in whole body protein breakdown of some 35%, as a result of the absence of any protein intake. Yet in Section 3 (Results 1) it was clear that after 3 weeks on Diet O, whole body protein breakdown was unchanged from normal. This pattern is confirmed by Figure 5.5A. However, if both (1-¹⁴C)leucine and (¹⁵N)glycine methods significantly underestimate true flux, then the breakdown pattern in Figure 5.5B may be more realistic.

When the large protein turnover changes resulting from prolonged protein-free dieting, were first measured with (1-¹⁴C)leucine (Section 3), it was planned that the (¹⁵N)glycine method, as well as being tested and developed itself, could be used to confirm these changes, and monitor their magnitude and time-course. However, the finding (with (1-¹⁴C)leucine) that some 2/3 of the decrease in whole body protein synthesis actually occurred in the first few hours of post-absorptive fasting, left only the small remaining decrease to be monitored over the 3 week period by the (¹⁵N)glycine method. Nevertheless, this method has confirmed the (1-¹⁴C)leucine results, not only showing the initial very rapid drop in protein synthesis, but indicating that after the second day without protein this decrease very rapidly tails-off, so that the total decline in protein synthesis has already been achieved after 1 week of protein-free diet.

Extending further the monitoring use of this method, the dependence of protein synthesis on the level of protein intake, as first suggested in Section 3, was further confirmed when the level of protein intake was varied whilst energy intake was kept constant (eg. Figure 5.6). In this obese subject, daily energy intake was maintained at 500 Kcal

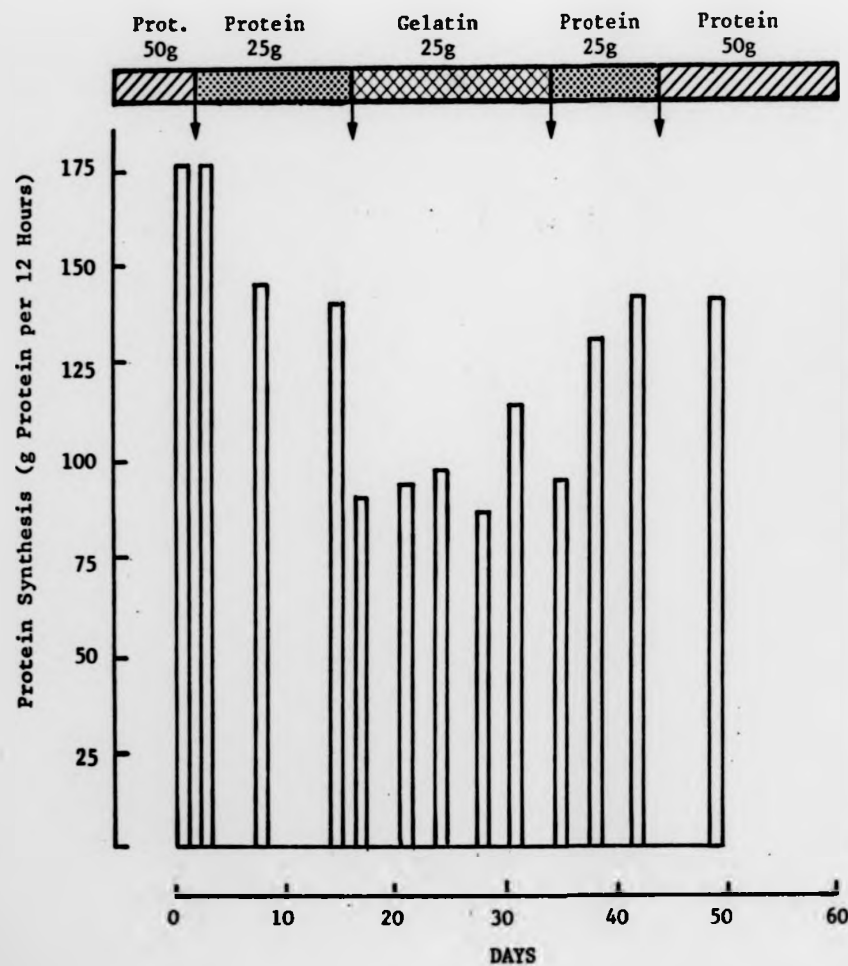


FIGURE 5.6.

Whole-body protein synthesis, measured by oral single dose (^{15}N)glycine, at different levels of protein intake, but with energy intakes maintained at 500 Kcal per day.

(Patient P.S.)

(2.1 MJ) whilst the quantity and quality of daily protein intake was varied, and protein turnover measured at frequent intervals by oral single dose (^{15}N)glycine using ammonia as urinary end-product. Despite the variability of results from this method, it is clear that whole body protein synthesis has dropped by almost 20% on the 25 g Protein diet, by approximately 45% on the gelatin diet, and then risen again with reintroduction of higher levels of utilisable protein.

The results from this section have thus confirmed the characteristics of whole body protein turnover initially established by the ($1\text{-}^{14}\text{C}$)leucine method, and further elucidated the relationship between protein synthesis and protein intake, including the time-course of changes.

A remaining uncertainty however, associated with both these methods of protein turnover measurement, is their accuracy in estimating true values of genuine precursor-pool flux. If the ($1\text{-}^{14}\text{C}$)leucine method underestimates flux by some 33% (a possibility discussed in Section 4) then this (^{15}N)glycine method would also appear to do so, considering the comparability of mean absolute values from both methods.

SECTION 6 ADAPTIVE AND ACUTE RESPONSES IN PROTEIN TURNOVER

RESULTS 4

DISCUSSION 4

RESULTS 4

This concluding group of protein turnover results brings together the concepts of adaption and long term change in protein metabolism from Section 3, with those of rapid diurnal change and acute sensitivity to protein intake, from Section 4.

The consequences of a protein-free 500 Kcal (2.1 MJ) per day diet for 3 weeks (Diet O) were shown to include a 40% drop in whole-body protein synthesis and an 85% drop in protein oxidation, measurements being made during the feeding 12 hours (Section 3). Section 4 showed that 2/3 of that drop in both synthesis and oxidation occurred within hours of post-absorptive fasting, as a diurnal change. Section 5 showed that the total 40 - 50% drop in synthesis was already achieved after some 6 days, and thereafter little further change took place, although once again these (^{15}N)glycine measurements represented feeding phase rates.

Two further outstanding questions required investigation:

- (1) After 3 weeks on the protein-free Diet O, is there any diurnal change in protein metabolism?
- (2) How does whole-body protein turnover respond to the sudden reintroduction of protein?

Each of the previous results sections have strongly suggested that the rate of whole body protein turnover is fundamentally dependent on the level of protein intake. In the light of these findings, there should be no diurnal change whatsoever in people with a daily dietary intake of only 500 Kcal of glucose syrup.

A group of 4 obese patients (Diet O-Diurnal group) were subjected to the 3 week dietary regimen of Diet O (500 Kcal (2.1 MJ) and 0 g protein per day), and after 20 days had their protein turnover measured for

30 - 36 hours by continuous (1-¹⁴C)leucine infusion. The measurement period thus included the daytime feeding 12 hours, the night-time fasting 12 hours, and a further 6 - 12 hours measurement period. During this last period one patient (T.D.) continued on Diet O, but the other 3 patients commenced Diet P which effectively kept the rate of energy intake unchanged (500 Kcal) but suddenly reintroduced protein (50 g protein in 12 hours).

Table 6.1 shows the mean diurnal rates of protein turnover for this group, initially on their 'Normal' Diet prior to the commencement of Diet O, and then after 3 weeks on the protein-free Diet O. The dietary and anthropometric data for the individual members of this group are given in Appendix 2.1 (the last 4 patients), and the individual protein turnover values are tabulated in Appendices 4.1 and 4.2.

TABLE 6.1. Mean (\pm S.D.) 24 hour protein turnover rates in 4 obese women, initially on 'Normal' Diet, and then after 3 weeks on Diet O. Measured by (1- 14 C)leucine infusion.

	Q	Z	B	E	I
	g Protein per 12 Hours				
<u>A. 'NORMAL' DIET</u>					
FEEDING	161.7	120.5	77.0	41.2	70.0
12 HOURS	±24	±22	±25	±7	±2.8
FASTING	112.0	92.0	112.0	20.0	C
12 HOURS	±28	±24	±28	±4	
% CHANGE (FEEDING → FASTING)	+30.0 %	+22.8 %	+62.5 %	+49.5 %	-
<u>B. DIET O</u>					
FEEDING	79.2	71.9	79.2	7.4	C
12 HOURS	±11	±10	±11	±2	
FASTING	76.7	70.4	76.7	6.4	O
12 HOURS	±10	±9	±10	±0.5	
% CHANGE	<u>NO SIGNIFICANT CHANGE</u>				

These results are unequivocal. Protein synthesis drops as expected with the change from feeding to fasting phase on the 'Normal' Diet, but on Diet O there is no diurnal change, whole-body protein synthesis rate remaining constant, depressed, and unaffected by the diurnal addition or subtraction of 500 Kcal of glucose syrup per 12 hours.

There is also very little diurnal change in protein oxidation rate on the protein-free Diet O regimen. The 50 - 60% drop in oxidation characteristic of the change from feeding to fasting phase on the 'Normal' Diet, has been replaced by a very low almost fixed oxidation rate, similar to obligatory protein oxidation.

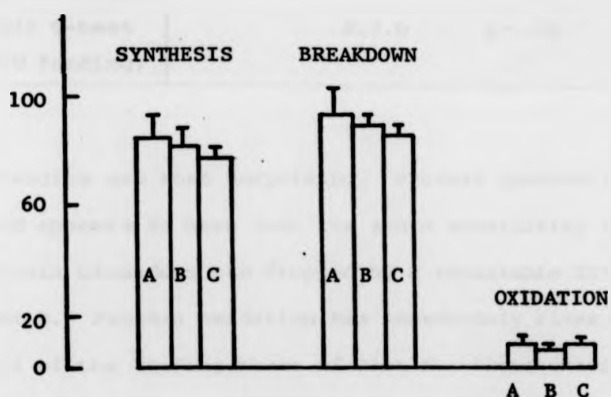
Protein breakdown, which increases immediately with the onset of post-absorptive fasting on the 'Normal' Diet regimen (here by 62.5%), not only loses its diurnal changes after 3 weeks on Diet O, but remains at a constant value that is neither depressed or elevated, but equal to the 'normal' feeding phase breakdown rate on the 'Normal' Diet.

For the one patient (T.D.) who then continued on Diet O whole-body protein synthesis continued to remain completely unaffected. This has already been shown in Section 4 (Fig. 4.2(b)), where hourly rates of protein synthesis extending over the three 12-hour phases of feeding, fasting and feeding were shown to lie on an almost horizontal regression line. Figure 6.1 shows how breakdown and oxidation rates also remain constant irrespective of the acute addition or subtraction of 500 Kcal of glucose syrup. (Once again the slight downward drift, which also occurs with continuous protein feeding over 24 hours, is likely to result from (^{14}C)leucine recycling.)

FIGURE 6.1. Protein Synthesis, Breakdown, and Oxidation rates over 3 consecutive 12-hour periods of feeding (A), fasting (B), and feeding (C), for patient T.D. on Diet O for 3 weeks.

PROTEIN SYNTHESIS

g Protein
per 12 Hrs



The other 3 patients of the group, after having their protein turnover measured over 24 hours on Diet O, were refed with protein (Diet P: 500 Kcal and 50 g protein over 12 hours) whilst keeping the energy intake at 500 Kcal per day. Table 6.2 shows the effect of this sudden reintroduction of protein.

TABLE 6.2. Mean (\pm S.D.) protein turnover rate for 3 obese women, during the first 12 hours of protein refeeding (500 Kcal/50 g protein per 12 hours), after having been on 500 Kcals/0 g Protein per day for 3 weeks.

	Q	Z	B	E	I
	g Protein per 12 Hours				
PROTEIN REFEEDING 12 HOURS	97.9 ± 12	74.1 ± 13	37.5 ± 11	23.8 ± 2	48.1
Matched-pair t-test (cf. Diet O Feeding)		N.S.D	$p < .02$	$p < .01$	

These results are most surprising. Protein synthesis remains depressed and appears to have lost its acute sensitivity to protein intake. Protein breakdown has dropped by a remarkable 51% from its value on Diet O. Protein oxidation has immediately risen to a value typical of the feeding phase of Diet P. These changes can be visualised better in perspective in Figure 6.2 (A) and (B), where the hourly values of synthesis, breakdown, and oxidation are monitored for one patient (E.K.), and the mean group values for each phase are also shown.

FIGURE 6.2.

(A) Protein Turnover continuously measured (Patient E.K.) showing the response to reintroduction of Protein.

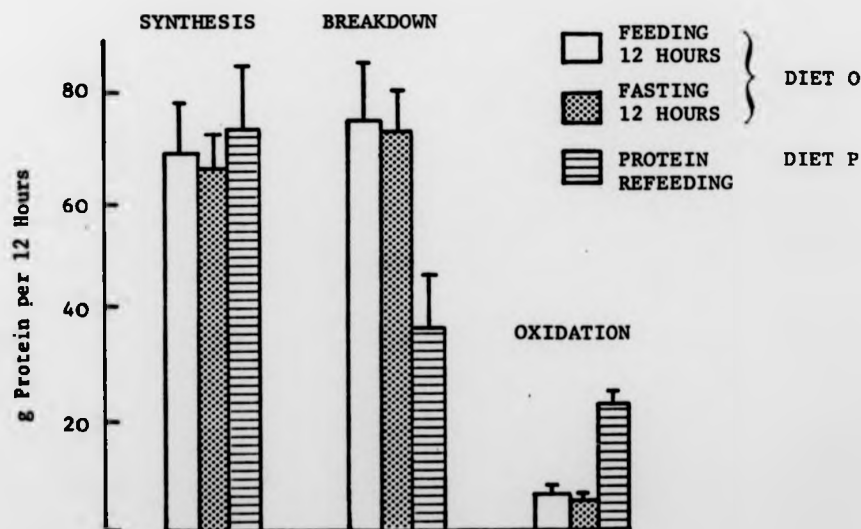
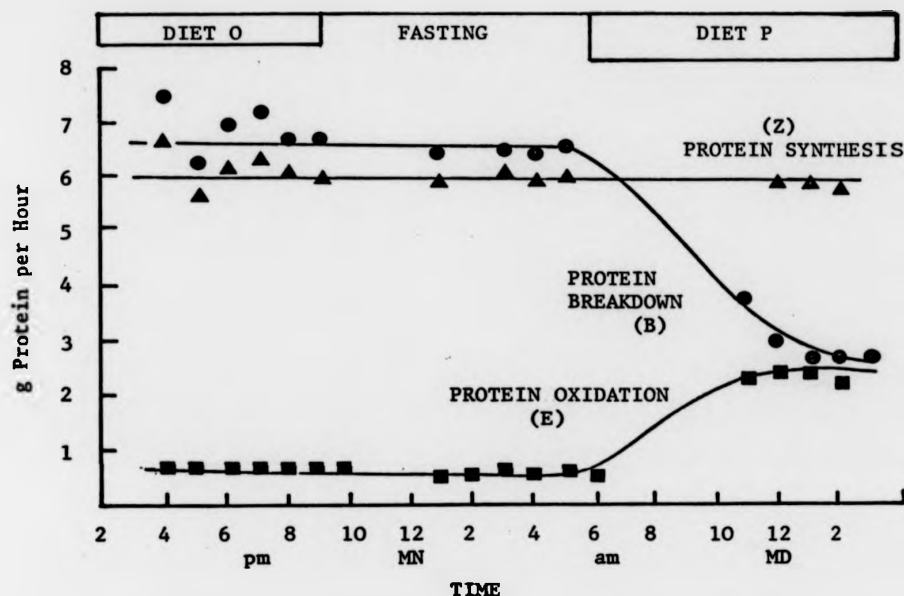


FIGURE 6.2.

(B) Values for Protein Synthesis, Breakdown, and Oxidation, over 3 consecutive 12 Hour periods of feeding (500 Kcal/0 Protein), Fasting, and Protein Refeeding (500 Kcal/50 g Protein).

DISCUSSION 4

The unexpected response of whole body protein metabolism to the reintroduction of protein after 3 weeks of protein-free diet, revives the question of whether a long term adaption has occurred leaving whole body protein synthesis in a suppressed and insensitive state.

On a normal dietary intake, it has been shown that protein synthesis is acutely responsive to the level of protein intake, with diurnal changes of almost 30% (Section 4). It has also been shown that following its initial rapid fall in response to normal post-absorptive fasting, protein synthesis continues to decrease if no protein is supplied in the diet (eg. total starvation, or Diet O), and the maximum 40 - 50% decrease in the rate of synthesis is achieved after about 6 days (Section 5). Perhaps it is whilst this final smaller slower decrease is occurring that the body's protein synthetic apparatus 'switches off' its sensitivity and acute responsiveness to protein intake. The remarkable failure of whole body protein synthesis to respond to the reintroduction of protein after a prolonged protein-free period, suggests a temporary adaption, possibly resembling hibernation. Its eventual increase with the reintroduction of a protein intake has been shown in Figure 5.6.

Protein oxidation may also have lost some responsive capability, despite its obvious immediate recovery with the reintroduction of dietary protein. Section 4 showed the large 60% diurnal drop in oxidation that typically occurs on a normal intake with the commencement of post-absorptive fasting. Three weeks on a diet of 500 Kcal/O g protein produced an overall 85% drop in oxidation, so that like protein synthesis, 2/3 of the total decrease in oxidation rate has occurred

immediately as an acute response to fasting, followed by a slower more gradual decrease over the next several days.

The absolute rate of protein oxidation after 3 weeks on Diet O, whether estimated by (1-¹⁴C)leucine infusion (13.7 g Protein per day) or from urinary-N excretion (2.8 g N per day, equivalent to 17.4 g Protein per day) represents a rate close to minimum obligatory protein oxidation and N excretion (eg. 2.4 g N/day, Calloway & Margen, 1971; Jourden et al, 1974). It is only half the rate of protein oxidation associated with prolonged total fasting, as is apparent from the data of Marliss et al (1978) who found a urinary-N excretion rate of 5.2 g N/day (equivalent to some 32.5 g protein) in a group of 7 obese subjects totally fasted for 3 - 4 weeks. Thus the presence of 500 Kcal of glucose syrup per day has induced significant protein sparing, consistent with the findings of Calloway & Spector (1954) who showed that negative nitrogen balance is progressively reduced by addition of non-protein energy up to (but not beyond) 700 Kcal/day.

However, it is interesting to note that despite the overall protein sparing effect of Diet O, and the large effect that glucose seems to have on protein metabolism when protein intake is zero, yet on a diurnal basis protein-synthesis, breakdown, and oxidation rates appear to be unaffected by 12 hour periods alternately on and off glucose, at least when protein metabolism is in a depressed 'adapted' state after 3 weeks of protein-free diet.

Probably the most remarkable immediate effect of the reintroduction of protein after 3 weeks on Diet O, is the huge drop in whole body protein breakdown - a drop of 51% in this group. Nor is this effect likely to be an artefact of the (1-¹⁴C)leucine method. It was shown in Section 4 that if the (1-¹⁴C)leucine method were corrected for its possible 33% underestimate of flux, then the 35% diurnal increase in

protein breakdown, seen on commencement of the post-absorptive fasting phase of the 'Normal' Diet, would be eliminated, and protein breakdown would then appear to show no change between feeding and fasting periods. But even if such a correction were applied to the flux rates obtained from both Diet O and Protein Refeeding phases, there would still be a very significant drop (30%) in protein breakdown as an immediate effect of reintroducing dietary protein.

Several recent studies have been concerned with the protein sparing effects of glucose, glucose plus amino acids, and amino acids alone, in either fasting or surgical patients. The mechanisms proposed, suggesting various changes in protein synthesis and breakdown, are mostly speculative, as the protein sparing has usually only been measured in terms of nitrogen balance data. A confusion of proposals and often erroneous assumptions can be seen even from some of the most celebrated studies.

Blackburn et al (1973) suggested that in starvation protein synthesis and breakdown both fall, but added amino acids spare protein by sustaining protein synthesis. They further suggested that addition of glucose to the amino acids is more harmful than amino acids alone, since glucose augments net protein breakdown by suppression of ketogenesis. In complete contrast, Jeejeebhoy (1977), Howard et al (1978), and Elwyn et al (1978) have all shown that the protein sparing achieved by glucose plus amino acids is greater than with amino acids alone, but whilst Howard et al suggested that the addition of glucose causes a decrease in protein breakdown, Elwyn et al proposed that it spares additional protein by increasing synthesis through the anabolic effect of insulin. Marliiss et al (1978) have claimed that during prolonged fasting protein breakdown decreases, and that the addition of dietary protein leads to increased protein

breakdown, so that there must be an even greater increase in protein synthesis to account for the observed protein sparing effect.

Despite this speculation on how the relative changes in protein synthesis and breakdown might balance to promote protein sparing, few attempts have been made to measure protein turnover in these circumstances. An exception is the study of Sim *et al* (1979) who showed that the additional protein sparing effect achieved by adding glucose to a constant intravenous amino acid regime, resulted from an increase in protein synthesis, with breakdown remaining unchanged.

Our data may contribute to an understanding of the mechanisms of protein sparing, since we have measured protein synthesis and breakdown in groups of subjects under the following conditions:-

- (a) prolonged fasting (see Section 5);
- (b) a hypocaloric glucose only intake (Diet O);
- (c) a hypocaloric carbohydrate plus protein intake (Diet P).

Table 6.3 sets out mean values of protein synthesis and breakdown, characteristic of prolonged exposure to these regimens.

TABLE 6.3. Whole body protein synthesis and breakdown in response to a prolonged period of either total fasting, 500 Kcal of glucose, or on 500 kcal/50 g Protein (Feeding Phase values are shown).

	PROTEIN SYNTHESIS Z g Protein per 12 Hours	PROTEIN BREAKDOWN B
Prolonged Fasting (1 week)	70	104
500 Kcal Glucose (Diet O: 3 weeks)	71	79
500 Kcal/50 g Protein (Diet P: 3 weeks)	115	84

These data suggest that in the absence of protein intake, glucose exerts its protein sparing effect by lowering protein breakdown, not by increasing synthesis. This is consistent with one of the basic findings in this thesis, that protein synthesis is largely dependent on (and acutely responsive to) the level of protein intake, and when the absence of protein is prolonged whole body protein synthesis falls to a low, constant, 'adapted' rate, unaffected by glucose intake, and only slowly responsive to protein intake. Thus the significant difference between obligatory minimum urinary-N excretion (about 2.4 g N/day) and the level of urinary-N excreted in prolonged fasting (about 5.2 g N/day), is entirely due to the higher rate of whole-body protein breakdown associated with prolonged fasting.

The differences between prolonged fasting values and Diet P values suggest that the protein sparing which is achieved by administration of both amino acids plus glucose to a fasting patient, results from the combined effect of an increase in synthesis and a fall in protein breakdown. However, in a patient who is already receiving an intake of amino acids only, the increased protein sparing benefit resulting from the addition of glucose is probably achieved by an increase in protein synthesis alone, as shown by Sim et al (1979), and discussed earlier in Section 3.

Finally, in a patient on a prolonged intake of glucose alone, there is a distinction between the mechanisms of acute and long term achievement of protein sparing, when amino acids are added. The initial effect is a dramatic drop in protein breakdown, the 'adapted' protein synthesis rate remaining unresponsive for several hours. Eventually protein synthesis rises to a level determined largely by the level of protein intake itself, but also influenced by the energy intake,

whilst protein breakdown returns to its usual level, seen during the feeding phase of most diets.

The remaining question extending from previous sections, is whether internal recycling, and the relationship between plasma and precursor-pool leucine, have changed after 3 weeks on Diet O, and therefore whether the leucine method still appears to give a 33% underestimate of flux.

In the following table (Table 6.4), since there is no protein intake, urinary-N excretion is assumed to represent the same 'whole body protein', containing 8.0% of leucine, that the leucine method represents in its estimate of protein oxidation rate.

TABLE 6.4. Comparison of protein oxidation rates, measured by urinary-N excretion and (1-¹⁴C)leucine methods, to calculate the possible underestimate of flux by the (1-¹⁴C)leucine method.

NAME	PROTEIN OXIDATION (g Protein/24 Hours)		$E_L/E_U \times 100 \%$
	Urinary-N excretion (E_U)	(1- ¹⁴ C)leucine (E_L)	
MW	14.7	12.1	82.3 %
TD	16.4	15.6	95.1 %
EK	19.2	15.4	80.2 %
RO	20.5	11.7	57.1 %
Mean			78.7 %
± S.D.			±15.8 %

These results have a wide scatter, and statistical comparison (matched pair t-test) with these patients' own 'Normal' Diet 'underestimate percentages' indicates no significant difference. Nevertheless, the results appear to suggest that under these conditions the

(1-¹⁴C)leucine method underestimates oxidation and flux by some 21%, a value almost identical with that obtained for E.E. (Section 3) and by Golden & Waterlow (1977).

On a normal dietary intake with feeding and fasting phases, in both obese and non-obese adults, the discrepancy between urinary-N based protein oxidation rates and those obtained from (1-¹⁴C)leucine indicated a consistent 33% underestimate by the leucine method. At lower rates of protein intake, and also in this group after 3 weeks on Diet O, the discrepancy seems to have lessened to about 21%. If these discrepancies genuinely indicate the underestimate of flux as measured by the (1-¹⁴C)leucine method, then it is possible that the relationship between plasma leucine disposal rate and precursor pool flux, may change with changing rates of protein intake. However, the apparent direction of this change (an increasing underestimate of flux with increasing protein intake) is such as to accentuate the changes in protein synthesis and oxidation shown to occur with changing diurnal and long term protein intakes.

SECTION 7 FURTHER COMMENTS, SUMMARY, AND CONCLUSIONS

The purpose of this section is to provide a summary of the results of the study and to discuss the implications of the findings.

The study was designed to investigate the effects of the independent variable on the dependent variable. The results of the study are summarized in the following table:

The results of the study indicate that there is a significant relationship between the independent variable and the dependent variable. The findings suggest that the independent variable has a positive effect on the dependent variable. The results are consistent with the hypothesis of the study. The findings have important implications for the field of study. The results of the study can be used to inform policy and practice. The findings suggest that the independent variable should be manipulated in order to achieve the desired outcome. The results of the study are a contribution to the knowledge of the field of study. The findings provide a basis for further research. The results of the study are a valuable addition to the literature. The findings are a significant contribution to the field of study. The results of the study are a valuable resource for researchers and practitioners alike. The findings provide a clear and concise summary of the study. The results of the study are a valuable addition to the literature. The findings are a significant contribution to the field of study. The results of the study are a valuable resource for researchers and practitioners alike. The findings provide a clear and concise summary of the study.

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FURTHER COMMENTS, SUMMARY, AND CONCLUSIONS

Two underlying aims have persisted through each section of this thesis - the development of the (1-¹⁴C)leucine method for whole-body protein turnover measurement, and the use of this method for the investigation of protein synthesis, breakdown, and oxidation, measuring both absolute values and change under different acute and long term dietary conditions.

Some remarkable, and hitherto unsuspected features of human protein metabolism have emerged, and most of these are summarised further below. However, one of the most significant of these is the acute sensitivity and responsiveness of protein turnover to any change in the level of protein intake. It was shown in Section 4 that when protein intake was either commenced or terminated, whole body protein turnover would respond within *one hour*. It became apparent that the hourly rate of protein turnover is dependent on the hourly rate of protein intake.

This finding, along with the measurement of substantial feeding/fasting diurnal changes in protein turnover, makes it clear that protein synthesis values, measured during a short period of constant feeding (or fasting), are not representative of genuine daily rates of protein synthesis (unless protein intake rate remains unaltered over the 24 hours). It is therefore usually inappropriate to express measured protein turnover rates as daily rates, unless measured over that period, or a prolonged measurement method is used (eg. (¹⁵N)glycine/urea end-product method). Estimated values for protein synthesis in g Protein/day are greatly influenced by diurnal feeding patterns.

A comparison of whole-body protein synthesis values from some previous studies, expressed in terms of g Protein per 12 hours

(appropriate for the ^{14}C -tracer studies which have all measured turnover under constant feeding conditions) shows where estimates by the $(1-^{14}\text{C})$ leucine method lie, in comparison with other tracer methods. The reasons for the probable overestimate by $(\text{U}-^{14}\text{C})$ tyrosine have been previously discussed (Sections 1 and 3). The $(1-^{14}\text{C})$ leucine results lie between the estimates from urea end-product and NH_3 end-product methods of (^{15}N) glycine, as discussed in Section 1.

TABLE 7.1. Whole body protein synthesis estimates by different measurement methods, for obese and normal subjects, on a normal diet.

STUDY	TRACER	SUBJECTS	PROTEIN SYNTHESIS g protein/12 Hour
James et al 1976	$(\text{U}-^{14}\text{C})$ tyr	Normal (6)	173
Sender et al 1975	$(\text{U}-^{14}\text{C})$ tyr	Obese (5)	200
O'Keefe et al 1974	$(1-^{14}\text{C})$ leu	Normal (4)	138
Steffee et al 1976	(^{15}N) gly/urea	Normal (6)	103
Young et al 1975	(^{15}N) gly/urea	Normal	102
Sim et al 1979	(^{15}N) gly/urea	Normal (5)	82
<u>THIS STUDY</u>			
$(1-^{14}\text{C})$ leucine I.V. infusion		Obese (19)	123
		Control (5)	112
(^{15}N) glycine/ NH_3			
I.V. infusion		Normal (4)	91
Oral multiple dose		Obese (6)	159
Single dose oral		Obese (6)	132
Single dose I.V.		Normal (6)	132

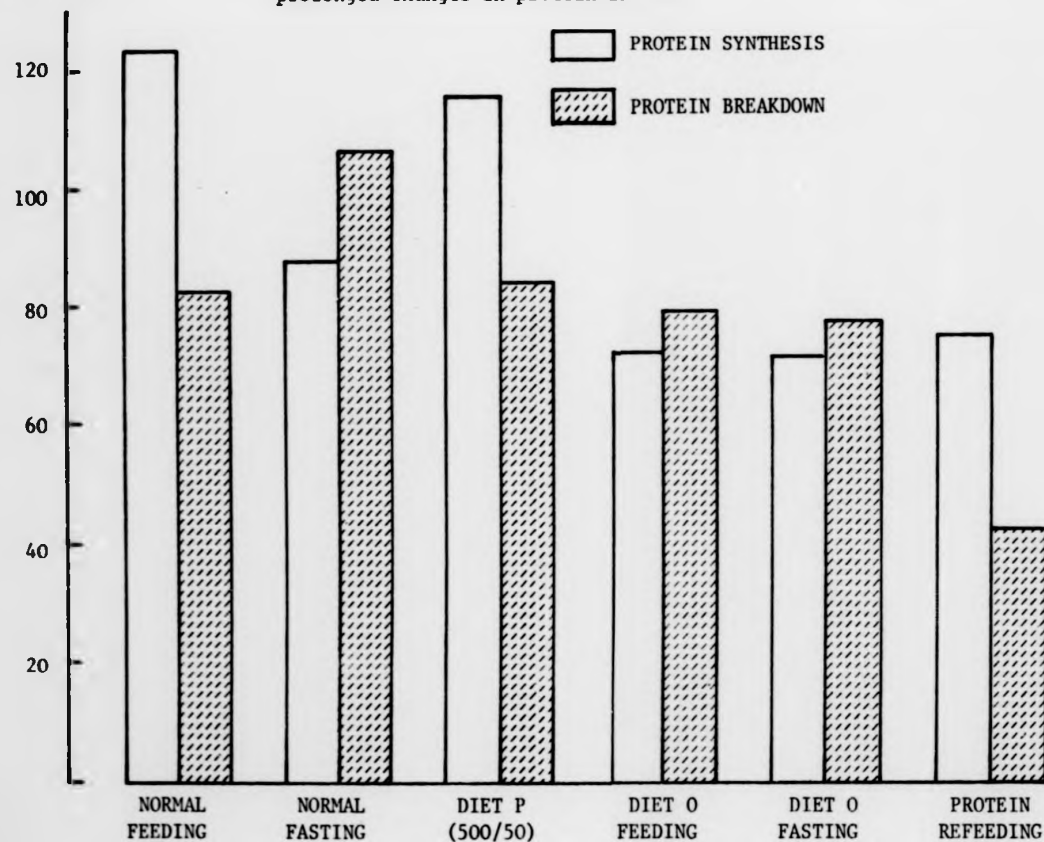
The values in Table 7.1 are representative of whole body protein synthesis under the influence of a constant protein intake (although there are some long gaps between feeds in the (^{15}N) glycine/urea end-product studies). However, the work presented in this thesis has

TABLE 7.2. Mean (\pm S.D.) absolute rates of protein turnover measured by (1-¹⁴C)leucine constant infusion method.

SUBJECTS	Number	INTAKE (over 12 Hours) Kcal/g Protein	SYNTHESIS Z	BREAKDOWN B	OXIDATION E
			g Protein per 12 Hours		
<u>NORMAL FEEDING</u>					
OBESSE GROUP	19	1712/70.5	122.9 \pm 17	83.0 \pm 17.5	44.6 \pm 8
CONTROL GROUP	5	1797/67.2	112.2 \pm 25	75.1 \pm 19	43.1 \pm 7
<u>NORMAL FASTING</u>					
OBESSE GROUP	10	0/0	87.6 \pm 15	106.3 \pm 17	18.7 \pm 4
CONTROL GROUP	5	0/0	81.6 \pm 15	100.5 \pm 15	18.9 \pm 4
<u>'DIET P' FEEDING</u> (after 3 weeks)	4	511/48.2	115.0 \pm 28	84.0 \pm 30	29.5 \pm 5.6
<u>'DIET O' FEEDING</u> (after 3 weeks)	4	495/0	71.9 \pm 10	79.2 \pm 11	7.4 \pm 2
<u>'DIET O' FASTING</u> (after 3 weeks)	4	0/0	70.4 \pm 9	76.7 \pm 10	6.4 \pm 0.5
<u>PROTEIN REFEEDING</u> (after 3 weeks Diet O)	4	529/48.1	74.1 \pm 13	37.5 \pm 11	23.8 \pm 2

g Protein
per 12 Hours

FIGURE 7.1. Whole body protein synthesis and breakdown, affected by acute and prolonged changes in protein intake.



accumulated a substantial number of (1-¹⁴C)leucine measurements of whole body protein turnover under a number of different dietary conditions and in both obese and normal subjects. The absolute values for these, as presented in the Sections 3 - 6, are set out in Table 7.2 and Figure 7.1.

When these data are considered in terms of the proportional changes that occur as a result of protein intake levels, the profiles of percentage change vary slightly depending on which level the changes relate to. In the previous sections, change has been expressed as a percentage of 'Normal' feeding phase values. These are summarised in Table 7.3.

TABLE 7.3. Mean protein turnover rates, expressed as percentages of 'Normal' feeding values (Obese patients only).

GROUP	SYNTHESIS Z	BREAKDOWN B	OXIDATION E
Normal Feeding	100 %	100 %	100 %
Normal Fasting	71.3 %	128.1 %	41.9 %
Diet P (500/50) Feeding	93.6 %	101.2 %	66.1 %
Diet O (500/0) Feeding	58.5 %	95.4 %	16.6 %
Diet O (500/0) Fasting	57.3 %	92.4 %	14.3 %
Protein Refeeding (500/50)	60.3 %	45.2 %	53.4 %

When the same results are expressed as a percentage of the normal post-absorptive fasting values, one can perceive in clearer perspective the effect of any level of protein intake, as well as the further adaptation that occurs as a result of prolonged absence of protein.

TABLE 7.4. Mean protein turnover rates, expressed as percentages of normal fasting (post-absorptive) values.

GROUP	SYNTHESIS Z	BREAKDOWN B	OXIDATION E
Normal Fasting	100 %	100 %	100 %
Normal Feeding	140.3 %	78.1 %	238.5 %
Diet P (500/50) Feeding	131.3 %	79.0 %	157.8 %
Diet O (500/O) Feeding	82.1 %	74.5 %	39.6 %
Diet O (500/O) Fasting	80.4 %	72.2 %	34.2 %
Protein Refeeding (500/50)	84.6 %	35.3 %	127.3 %

Thus if post-absorptive fasting is regarded as 'baseline', the effect of normal feeding is a dramatic 40% increase in protein synthesis, a 139% increase in oxidation, and a 22% drop in breakdown. However it is the profiles of synthesis, breakdown and oxidation, and how these change with protein intake, that are remarkable. The adaption and non-responsiveness of protein synthesis to reintroduction of protein, the adaption of protein breakdown with an even further amazing decrease when protein is reintroduced, and the enormous changes in protein oxidation are all apparent particularly from Table 7.4.

It is possible that the absolute values of protein synthesis, breakdown and oxidation, shown in Table 7.2, may all be significantly too low. In Sections 3,4, and 6, using a method of comparison similar to that suggested by Golden & Waterlow (1977), it was shown that the (1-¹⁴C)leucine method appeared to underestimate oxidation rates by about 20%, and that if it were assumed that oxidation and synthesis shared the same precursor pool, then it was possible to quantitate this error of underestimation for flux, synthesis and breakdown.

In fact the precursor pools for whole body protein synthesis and leucine oxidation are likely to be quite different, since it appears that leucine oxidation primarily occurs in muscle (Elia *et al*, 1980). Furthermore in any tissue the precursor pool from which an amino acid is incorporated into protein (eg. perhaps in the cell membrane, functionally somewhere between plasma and intracellular pools. Airhart *et al*, 1974) may be structurally and functionally very different from the precursor pool from which its oxidation occurs.

Nevertheless, if the correction factors calculated for the possible underestimate of flux by (1-¹⁴C)leucine are applied, then the absolute values previously shown in Table 7.2, will be increased. These 'corrected' rates of synthesis, breakdown and oxidation are shown in Table 7.6 further below.

The correction factors themselves appear to be partly dependent on the level of protein intake, or perhaps flux. The relationship between plasma and 'precursor pool' does not seem to alter with acute diurnal changes in protein intake (Section 4), since it was shown that on the Normal Diet regimen, the underestimate of flux in 15 obese and control subjects remained at approximately 33%, irrespective of feeding and fasting. When the rate of protein intake was only half that of the 'Normal' Diet (eg. for patient E.E., and the geriatric patients of Golden & Waterlow, 1977), the underestimate improved by decreasing to approximately 20%. A similar underestimate was found for the 'adapted' subjects after 3 weeks on Diet O. Thus a possible 'slow response' relationship may link the rate of protein intake (and hence flux) with the level of underestimation of flux by the (1-¹⁴C)leucine method.

TABLE 7.5. Percentage underestimate of 'true' flux by the (1-¹⁴C)leucine method, at different rates of protein intake.

STUDY	PATIENTS	PROTEIN INTAKE g Protein/hour	% UNDERESTIMATE of FLUX
Golden & Waterlow (1977)	6 Geriatric	2.1	23 % ± 6
SECTION 3	Patient E.E. Obese	3.0	21 %
SECTION 6	4 Obese	0	21 % ± 15
SECTION 4	5 Normal	5.6	35 % ± 15
	10 Obese	6.1	33 % ± 7
	Normal Feeding - (JKG)	5.6	31 %
	Normal Fasting -	0	32 %

TABLE 7.6. Mean absolute rates of protein turnover, measured by (1-¹⁴C)leucine, and 'corrected' for the method's underestimation of flux.

SUBJECTS	PERCENT UNDERESTIMATE	SYNTHESIS	BREAKDOWN	OXIDATION
		Z g Protein per 12 Hours	B	E
Normal Feeding	33 %	183	165	67
Normal Fasting	33 %	131	159	25
Diet P Feeding	33 %	172	65	44
Diet O Feeding	21 %	91	100	9
Diet O Fasting	21 %	89	97	8
Protein Refeeding	21 %	94	63	30

These corrections have not affected the proportional changes that occur in synthesis and oxidation in response to dietary protein, but the response of protein breakdown appears to be quite altered. It now appears that diurnal post-absorptive fasting no longer causes an increase in protein breakdown, which remains constant.

Furthermore, both Diet P and Diet O now appear to drastically reduce protein breakdown, whereas in previous tables they had no effect. Three weeks on 500 Kcal/50 g protein drops breakdown by 60%, whilst there is a 38% decrease after 3 weeks of the protein free diet. The sudden further drop in breakdown is still seen however, when protein is reintroduced after its prolonged absence from the diet.

The argument can only remain unresolved at present, on whether these corrections are valid or necessary. Fortunately they leave the relative changes in protein synthesis and oxidation unaffected, but uncertainty about both the absolute values of protein turnover, and the response of protein breakdown to protein intake, will remain, at least until other techniques or independent comparisons reveal more of the functional relationships between plasma, intracellular and precursor pools.

It remains to summarise the findings and conclusions, for both methodology and protein metabolism, that have been presented in the previous sections.

The (1-¹⁴C)leucine constant infusion method has been central to most of the measurements presented. The development of the method's most critical techniques has been described in Section 2, particularly showing how a system was built to enable prolonged protein turnover measurements. This capability allowed the investigation of diurnal patterns of protein turnover, and prompted the energy metabolism measurements shown in Part B.

Two sources of error with the (1-¹⁴C)leucine method, and their appropriate correction factors, have been investigated. In Section 2 it was shown that whenever oxidation is measured from the decarboxylation of a (1-C) labelled tracer amino acid, a 10% loss of labelled CO₂ needs to be accounted for. It was shown with 10 (1-¹⁴C)bicarbonate infusions,

that the correction factor (ie. 100/90) remains constant for at least 36 hours of infusion, and is unaffected by diet or diurnal feeding/fasting changes.

The second source of error (the known underestimate of flux by the (1-¹⁴C)leucine method, because of internal recycling) and the validity of its attempted quantitation, has been discussed in every section. It appears that the underestimate may increase with increasing protein intake or flux, so that on a normal dietary intake of some 70 g protein given over 12 hours, the underestimate was 33% in both obese and non-obese subjects, whilst at lower rates of protein intake or even protein-free diets the underestimate was 21%. However, it has been pointed out that it is dubious whether these underestimates really apply to the precursor pool for protein synthesis.

In Section 5, the (¹⁵N)glycine method, using ammonia as the excreted end-product, and with a number of different dose-administration techniques, proved to give reasonable comparative estimations of protein turnover with the (1-¹⁴C)leucine method, although individual values were often widely scattered. On normal protein intakes, the ¹⁵N method usually gave marginally higher estimates of flux and synthesis than the (1-¹⁴C)leucine method, but on prolonged protein free diets, its estimates were usually lower. If the (1-¹⁴C)leucine method underestimates flux, then the (¹⁵N)glycine/ammonia end-product would also appear to do so. The outstanding advantage of this method, particularly with single dose administration, was its ability to make repeated frequent measurements of protein turnover. It was shown that such measurements could be made within 9 hours of each other, and in addition long term monitoring of protein metabolism could be achieved.

Although it was originally intended to investigate some general aspects of human whole-body protein turnover, the unexpected findings

that first emerged, and the direction of research they prompted, have orientated the results about a fundamental relationship in protein metabolism - the close dependence of protein turnover on protein intake. As this close dependence and its sensitivity have been explored in each section of this thesis, a number of remarkable findings and conclusions have emerged, and the most notable of these are summarised here.

1. Whole body protein synthesis is largely dependent on protein intake. When protein intake is above requirement protein synthesis remains normal. When intake falls below requirement, protein synthesis rate falls, and may fall some 40 - 50% below normal with prolonged protein free diets or starvation.
2. Increasing the rate of energy intake, up to approximately 40 Kcal/hour, will improve the rate of protein synthesis if this is already low because of an inadequate protein intake. Further addition of energy (above 40 Kcal/hour) has little effect. In this way, marginally inadequate protein intakes may become adequate, with the addition of extra energy up to 40 Kcal/hour.
3. There are two rates of response of whole-body protein synthesis to changes in protein intake:
 - (a) An acute rapid response (3 - 4 hours), accounting for almost 2/3 of the total eventual change in synthesis rate, and immediately reversible.
 - (b) A slow long term 'adaptive' response in which protein synthesis rate gradually approaches a corresponding 'adapted' level (over 5 - 6 days) if the changed rate of protein intake is maintained. This is not an immediately reversible change.

4. There are large diurnal changes in whole body protein turnover, resulting from acute changes in the rate of protein intake. Within hours of commencement of normal post-absorptive fasting, whole body protein synthesis falls by 27%, oxidation by 60%, and breakdown may increase by some 35%. These rates return to the original levels within hours of recommencing a normal protein intake.
5. After several days on a low protein diet, whole-body protein synthesis falls to its own correspondingly decreased level. With an energy intake maintained at 40 Kcal/hour, an intake of 4 g Protein/hour still maintains normal feeding phase protein synthesis, 2 g Protein/hour causes an eventual fall of 20% in protein synthesis, and at 40 Kcal/0 g Protein per hour protein synthesis eventually falls some 40% below normal (after 6 days). Total continuous fasting leads to a fall in synthesis close to 50%.
6. In its 'adapted' state after a prolonged protein-free diet, whole-body protein synthesis is depressed and unresponsive to the reintroduction of protein. However protein breakdown rapidly responds, temporarily dropping to an extraordinarily low level, some 54% below its normal feeding rate, and 65% below its normal fasting rate.
7. There are no intrinsic circadian rhythms in whole body protein turnover.
8. Plasma free amino acid pools change acutely with acute changes in amino acid intake. With a constant leucine intake (normal diet) the plasma free leucine pool was found to remain constant (148 μ moles/litre in obese;

102 μ moles/litre in non-obese), but with post-absorptive fasting the plasma free leucine dropped by 30% and remained there.

9. There is little difference in protein turnover rates between obese and normal subjects. Although on a normal diet the lean subjects were shown to have marginally (though not significantly) slower whole-body protein synthesis rates (*lean*: 194 g Protein/24 hours; *obese*: 210 g Protein/24 hours), the relative diurnal changes that occurred in synthesis, breakdown and oxidation were identical for both groups.
10. The mechanisms of protein-sparing, in terms of protein synthesis and breakdown have been suggested. Compared with the prolonged fasting state, the addition of glucose decreases protein breakdown but leaves protein synthesis in its depressed condition. The addition of amino acids alone increases protein synthesis and decreases breakdown to its normal feeding level. Addition of glucose to that same intake of amino acids increases protein synthesis further, leaving breakdown normal.

There are many interesting aspects of protein turnover, and other side issues, that have arisen during the course of this work, that beg further investigation. Indeed some related studies have already been undertaken such as measuring the effect of severe infection (meningitis) in children, and vaccination fever in adults, on protein synthesis. However, two areas of work appear to be outstanding in their need for further research. These include a better definition of the dependence of protein synthesis on protein intake, and particularly the effect of

small quantities of non-protein energy, on this relationship.

The other main area is the uncertainty remaining about the precursor pool for protein synthesis and its relationship to plasma and oxidation precursor pools, the size of the underestimate of flux that results from sampling (1-¹⁴C)leucine from plasma, whether plasma/precursor pool relationships really change with protein intake, and the contribution of individual organs and tissues to whole body protein turnover under different conditions.

Work is already progressing on this second group of problems. In a current collaborative study eight infusions with the double-labelled stable isotope (1-¹³C, ¹⁵N)leucine have been carried out and muscle biopsies taken at both beginning and end of plasma plateau period. Half the infusions were done under feeding conditions, and half during prolonged fasting. The aim is not only to compare protein turnover results from a simultaneous plasma specific activity method and an excreted end-product method using the same amino acid, but to compare muscle protein synthesis rates (from incorporation of label) with whole body protein synthesis, examine the relationship between plasma and intracellular free leucine pools, and determine how these relationships change with feeding and fasting. (With such dedication to protein turnover research as shown by collaborator M.J.R., enjoying his 8th muscle biopsy ... what cannot be achieved?)



Colleague M.J.R. - enjoying his 8th muscle biopsy.

The (1-¹⁴C)leucine method has therefore been used not only in exploring the nature of whole-body protein turnover, but also in promoting the development of other tracer methods such as the (¹⁵N)glycine/NH₃ end-product method, and more recently the (1-¹³C)leucine method.

It is hoped that through the exploitation of the (1-¹⁴C)leucine method, and the protein turnover measurements and findings that have been presented in this thesis, a small but meaningful contribution has been made towards our understanding of the enormous complexity of human protein metabolism.



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The ($1-^{14}\text{C}$)leucine method has therefore been used not only in exploring the nature of whole-body protein turnover, but also in promoting the development of other tracer methods such as the (^{15}N)glycine/ NH_3 end-product method, and more recently the ($1-^{13}\text{C}$)leucine method.

It is hoped that through the exploitation of the ($1-^{14}\text{C}$)leucine method, and the protein turnover measurements and findings that have been presented in this thesis, a small but meaningful contribution has been made towards our understanding of the enormous complexity of human protein metabolism.

PART B

ENERGY METABOLISM

SECTION 8 ENERGY METABOLISM

8.1. INTRODUCTION

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8.5. SUMMARY AND CONCLUDING COMMENTS

8.1. INTRODUCTION

A system has been developed, based on whole-body protein turnover measurements and indirect calorimetry, by which the actual quantities of protein, carbohydrate, and fat being oxidised for energy expenditure, mobilised from stores, or deposited into stores, can be estimated on a whole-body basis, and even monitored hourly, under most conditions.

Calculation of the 'metabolic mixtures' of fuels being utilised have been made in some past studies - particularly notable are the early calculations of Zuntz & Schumburg (1901); Lusk (1924); and Weir (1949). However, even up to the present time, most of these estimations have been limited by methodology and restricted by the assumption that indirect calorimetry is only valid under conditions of overall oxidation. These limitations have meant that the few studies attempting analysis of metabolic mixtures have usually been under fasting or heavy exercise conditions (eg. the marathon runner; Edwards (1934)), and are probably the main reasons why indirect calorimetry is usually aimed at short term measurements of total energy expenditure, overall energy balance, and comparisons with direct calorimetry.

This section shows how these restrictions have been largely overcome. By using a system which enabled the continuous measurement of protein oxidation, oxygen uptake and carbon dioxide output over 24 hours or longer, many of the methodological limitations and their associated errors have been bypassed. Furthermore, by analysing the stoichiometry of the oxidation of fuel constituents (ie. amino acids, fatty acids - factors unavailable to the earlier studies), and adopting a stochastic 'whole-body' approach to the intake and output of carbon and oxygen, formulae have been developed that allow the

estimation of rates of utilisation or synthesis of fuels, even on a hourly basis, and diurnal changes in body fuel stores. It appears that this has not been attempted previously.

8.2. BACKGROUND

It was Zuntz & Schumburg (1901) and then Lusk (1928) who originally established the method for calculating the metabolic mixture of oxidation. This involved the measurement of total O_2 consumption, CO_2 output and urinary N output and then working through a multi-step procedure (Brody, 1945). This meant initially calculating and subtracting the contribution of protein oxidation from O_2 consumption and CO_2 production, then calculating from this non-protein O_2 and CO_2 the proportional oxidation of fat and carbohydrate. Finally by using tables (Lusk, 1924) or diagrams (Michaelis, 1924) of estimated caloric equivalents for non-protein O_2 consumption, the caloric contribution of each of the fuels was estimated.

For most studies this was the desired end-product of the measurements and there seems to have been little interest in calculating actual quantities of fuels oxidised, although this simply involved the use of caloric values for fuels in mixed diets, the 'standard values' of Rubner (1901) (4.1 Kcal/g for both carbohydrate and protein, and 9.3 Kcal/g for fat) being most commonly used. Benedict (1907) was the first to use this method to determine the quantity of glycogen oxidised in seven men during seven days of fasting.

Weir (1949) showed that it was unnecessary to use caloric equivalents of O_2 for fat and carbohydrate or their percentage heat, as given in the standard tables, and both he and later Consolazio et al (1963) derived formulae for fuel quantities in the metabolic mixture, based on the contribution of protein, carbohydrate and fat to O_2 consumption and CO_2 output, having measured protein oxidation from urinary-N excretion. It is this method, either in its original form, or as modified by Weir or Consolazio et al, that is still used for

calculation of the utilisation of fuels (eg. Kerr et al, 1978; Bursztein et al, 1980; Ravussin et al, 1980).

If these formulae, based on the original work of Zuntz, Lusk, and Benedict, and modified by Weir and Consolazio, are valid, why have there been so few studies attempting to measure fuel utilisation or investigate changes in body stores? Contributing reasons have probably included the recognised inaccuracies resulting from the usual techniques of short-term indirect calorimetry (eg. Douglas bag), and known discrepancies between these and simultaneous direct calorimetric measurements (Webb et al, 1980A).

However, even if technical difficulties could be eliminated, it appears that the single most convincing objection to the use of indirect calorimetry, irrespective of its purpose, has been the underlying belief that R.Q. values only have validity between 0.71 and 1.00, and even then only when oxidative metabolism far exceeds all other. It is argued that because R.Q. values were originally calculated only from the oxidative stoichiometry of protein, carbohydrate, and fat, they are therefore only relevant to oxidative metabolism, and obviously any significant occurrence of other metabolic pathways such as fat synthesis, with its totally different O_2/CO_2 relationship (eg. the production of palmitate from glucose has an R.Q. of 2.75! McGilvery, 1979), will destroy any measurable relationship between O_2 uptake, CO_2 output, and whole body fuel utilisation.

This warning, concerning the limited validity of indirect calorimetry, was given by Brody (1945) who felt that,

"There is no doubt that the indirect method (of calorimetry) is not rigorously sufficient, as when oxygen is used in the body for purposes other than oxidation, such as massive conversion of carbohydrate containing some 53% O_2 to fat containing 11 - 12%."

It is interesting to note that both Lusk (1928A) and Benedict (1937) thought that R.Q. values above 1.0 *were* completely valid, and undertook overfeeding experiments to demonstrate this, achieving R.Q. values up to 1.5, and then using the usual method of calculation to estimate the quantity of carbohydrate converted to fat.

Nevertheless, it is now usually accepted that the validity of indirect calorimetry is limited. McGilvery (1979) reviews the biochemical processes such as fat synthesis from carbohydrates, the Cori cycle, and interconversion of amino acids, each of which has a gas exchange and respiratory quotient of its own, and concludes that,

"... the use of calorimetry for the assessment of oxidative metabolism is of value only under two circumstances: when the rate of oxidative metabolism far exceeds the rate of synthetic processes, and when the rate of amino acid metabolism is relatively low, compared to the rate of fat and carbohydrate metabolism."

In the following section, using the basic stoichiometric relationships of gas exchange and fuels, and using a stochastic approach in which gas exchange relationships for the many individual biochemical pathways can be ignored, equations are derived from 'first principles' for calculating the quantities of carbohydrate and fat metabolised.

The validity of these equations is reinforced by re-deriving them from a basic C, N, O, H, and S 'balance' approach, and arriving at identical formulae.

When tested theoretically against possible or hypothetical metabolic situations where protein, carbohydrate and fat are utilised or deposited in many different combinations (eg. massive synthesis of

fat from carbohydrate and simultaneous oxidation of protein), including situations where R.Q. values are above 1, these equations appear to give valid quantitative answers for fuel mobilisation or deposition.

It appears therefore, as suggested and tested by both Benedict, and Lusk (1928), that the relationship between O_2 and CO_2 at whole-body level does not suddenly become meaningless when synthetic processes predominate. Indeed, since carbon and oxygen cannot disappear, but must be ultimately stored or used and excreted, from a stochastic or 'balance' viewpoint net synthesis is simply mathematically equivalent to negative net utilisation of any fuel.

These formulae, including a term for protein oxidation as measured from protein turnover measurements, are used to measure 24 hour fuel deposition and utilisation patterns in both obese and non-obese normal subjects, on different patterns of dietary regimes. By taking hourly measurements of all three quantities (g Protein /hour, moles O_2 uptake/hour, and moles of CO_2 output/hour) and using a programmable calculator, the quantities of each of the fuels being utilised or synthesised each hour have been calculated. These combined with fuel intake values, can then give hourly profiles of net fuel deposition and mobilisation patterns. The results, showing these diurnal profiles of whole body fuel economy, are remarkable.

8.3. DERIVATION AND TESTING OF THE FORMULAE

8.3.1. Derivation I

The first method of deriving the formulae is based on the assumption that total O_2 uptake and CO_2 output are solely the result of metabolism of protein, fat, and carbohydrate. Consolazio (1963A) used this approach, but assumed there was no food intake and oxidation was predominant. However if the quantities of oxygen and carbohydrate contributed by food are known, and it is assumed that any significant storage is in protein, carbohydrate and fat stores, then the following relationships should hold irrespective of whether net oxidation or synthesis is occurring:

$$\text{Total } O_2 \text{ intake} = O_{2P} + O_{2C} + O_{2F} \quad (\text{in moles } O_2)$$

$$\text{Total } CO_2 \text{ output} = CO_{2P} + CO_{2C} + CO_{2F} \quad (\text{in moles } CO_2)$$

The subscripts P, C, F, refer to protein, carbohydrate and fat.

It is the exact composition and quantity of each of the fuels being oxidised (or synthesised) that will determine stoichiometrically the quantity of O_2 or CO_2 associated with each of the fuels. Thus for any particular fuel, whether from food or body stores, a constant can be determined stoichiometrically, relating the fuel to O_2 uptake and CO_2 output. Hence it is designated that:

P = g Protein metabolised

C = g Carbohydrate metabolised

F = g Fat metabolised

then a series of fuel-specific constants can be determined so that the previous equations can be rewritten:

$$\text{Total O}_2 \text{ (moles)} = aP + bC + cF \quad \text{Equation 8.1}$$

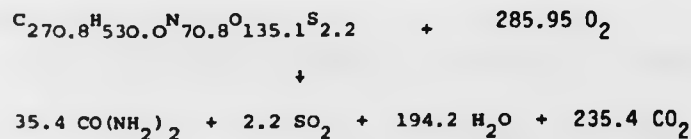
$$\text{Total CO}_2 \text{ (moles)} = xP + yC + zF \quad \text{Equation 8.2}$$

where the constants a, b, c, x, y, z , are in terms of moles.g⁻¹ and all depend on the stoichiometric composition of the protein, fat, and carbohydrate being metabolised.

When these formulae were initially derived, the rather laborious process of working out the values of these constants was undertaken, for each of the diets used, as well as for fasting. This was felt necessary because it was quite unknown whether meaningful differences in O₂ uptake and CO₂ output were likely to result from the metabolism of the markedly different proteins and triglycerides consumed in the diet, or mobilised from body stores. Nor was it known whether these were likely to differ significantly from the traditionally used values, derived empirically by Zuntz (1897) or Magnus-Levy (1907) or others.

The method of calculation of a, b, c, x, y , and z for the milk-based 'Normal' Diet is shown below.

The amino acid pattern for milk 'protein' (from Paul & Southgate, 1978) was used to calculate the empirical 'protein' associated with 1 g of N in milk (this pattern is set out in Appendix 5.1). The stoichiometry of its complete oxidation to urea was then used to calculate the quantities (in millimoles) of O₂ and CO₂ involved, ie.



This gives an R.Q. of 0.823, and shows that 1 g N in milk (\approx 6.38 g Protein) is associated with 0.2860 moles O_2 and 0.2354 moles CO_2 . Thus the calculated values of a and x are:

$$a = 0.0448 \text{ moles } O_2/\text{g milk protein}$$

$$x = 0.0369 \text{ moles } CO_2/\text{g milk protein}$$

A similar approach was used for milk fat. The relative proportions of the main fatty acids (and glycerol) in milk (Paul & Southgate, 1978) were used to calculate a 'representative triglyceride' (see Appendix 5.2), and this was used to calculate stoichiometrically the O_2 and CO_2 associated with its complete oxidation. Thus for the milk 'triglyceride' $C_{13.3}H_{26.55}O_{2.25}$, with an R.Q. of 0.707, the calculated values of b and y are:

$$b = 0.0845 \text{ moles } O_2/\text{g milk fat}$$

$$y = 0.0598 \text{ moles } CO_2/\text{g milk fat.}$$

Milk carbohydrate, expressed in terms of glucose (rather than lactose) yielded the following stoichiometric values of c and z :

$$c = 0.0333 \text{ moles } O_2/\text{g milk glucose}$$

$$z = 0.0333 \text{ moles } CO_2/\text{g milk glucose.}$$

By resubstituting these values into Equations 8.1 and 8.2, the appropriate formulae for calculating quantities of glucose and triglyceride oxidised can be derived, by solving the simultaneous equation below.

$$\text{Total } O_2 \text{ (moles)} = 0.0448 P + 0.0333 C + 0.08455 F$$

$$\text{Total } CO_2 \text{ (moles)} = 0.0369 P + 0.0333 C + 0.05977 F$$

Hence on a milk based diet:

$$\text{GLUCOSE OXIDISED g} = 102.483 (\text{CO}_2\text{moles} - 0.7070 \text{ O}_2\text{moles} - 0.0052 \text{ Protein g})$$

$$\text{FAT OXIDISED g} = 40.365 (\text{O}_2\text{moles} - \text{CO}_2\text{moles} - 0.0079 \text{ Protein g})$$

This same procedure can therefore be used for deriving fuel utilisation equations, for any diet or for fasting. By working through the amino acid composition, triglyceride/fatty acid composition, and carbohydrate components of the fuels being utilised, a series of constants, *A, B, C, D, E*, (see Table 8.1) can be calculated specific to any diet, for substitution into the general formulae below (Equations 8.3 and 8.4). Thus the quantities of carbohydrate and fat being utilised at any moment, can be computed, depending on O_2 uptake, CO_2 output, and an independent measurement of protein oxidation.

The following general formulae, associated with the diet-specific constants *A, B, C, D*, and *E*, were used with O_2 uptake and CO_2 output expressed in moles, and Protein, in grams, so that the calculated quantities of glucose and fat were also in grams. In the 'work-sheet', devised by Consolazio *et al* (1963), which amounts to a procedure similar to the formulae below, the term for protein oxidation is obtained by the traditional N excretion measurement. In our own patients, it was measured using the $(1-^{14}\text{C})$ leucine method.

$$\text{GLUCOSE UTILISED} = A(\text{CO}_2 - B \text{O}_2 - C \text{ PROTEIN})$$

Equation 8.3

$$\text{FAT UTILISED} = D(\text{O}_2 - \text{CO}_2 - E \text{ PROTEIN})$$

Equation 8.4

The associated values of A , B , C , D , and E , specific for each diet, or for fasting, are set out in Table 8.1. Also calculated into these terms are the values derived from the 'standard' calorimetric data of Zuntz (1897), and the slight modification of the protein factor (C) by Lusk (1928), for O_2 and CO_2 exchange associated with metabolism of fuels in the human body.

The calculated answer from Equation 8.3 will be in terms of glucose utilised (rather than glycogen, starch, or other di- or polysaccharide). This is because for each of the dietary situations the carbohydrate being metabolised was converted to its equivalent in 'available monosaccharide'. The conversion factors for polysaccharides and their application to the polysaccharide syrup Hycal (Diet O) are shown in Appendix 5.3.

There are, of course, some underlying assumptions for both the use of the general equations, and the analyses of the actual amino acids and fatty acids being metabolised, and these will introduce some error. The assumptions associated with the formulae are largely those of indirect calorimetry, but include the accuracy of O_2 and CO_2 measurement, and how representative such measurements are of 24-hour values. These were discussed earlier in Section 2. The equations also assume that biochemical pathways not associated with fuel metabolism (ie. other than oxidation, synthesis, interconversion), contribute and produce negligible quantities of O_2 and CO_2 .

TABLE 8.1. Numerical values of the factors A, B, C, D, and E, for Equations 8.3 and 8.4, for the metabolism of fuels of different composition. (P) the 'Mean Amino Acid' per mole of N, and (F) the 'Mean Triglyceride' are shown for each diet.

DIET	A	B	C	D	E
<u>'NORMAL' DIET A (milk-based)</u>	102.483	0.707	0.0052	40.365	0.0079
P: $C_{3.82}H_{7.49}N_{1.91}S_{0.03}O_{0.03}$					
F: $C_{13.3}H_{26.55}O_{2.25}$					
<u>'NORMAL' DIET B (cheese & crackers)</u>	102.902	0.708	0.0052	40.512	0.0078
P: $C_{3.81}H_{7.35}N_{1.91}S_{0.05}O_{0.05}$					
F: $C_{13.41}H_{26.62}O_{2.25}$					
<u>FASTING</u>	104.355	0.712	0.0040	41.066	0.0063
P: $C_{3.33}H_{6.78}N_{1.79}S_{0.04}O_{0.04}$					
F: $C_{13.61}H_{26.46}O_{2.23}$					
<u>'DIET O' (HYCAL)</u>	104.355	0.712	0.0040	41.066	0.0063
P: $C_{3.33}H_{6.78}N_{1.79}S_{0.04}O_{0.04}$					
F: $C_{13.61}H_{26.46}O_{2.23}$					
Zuntz (1897)	91.69	0.711	0.0045	38.31	0.008
Lusk (1928)	91.69	0.711	0.0041	38.31	0.008

The validity of the calculated sets of factors for each diet depends on the assumption that the diet being consumed at a particular time accounts for total fuel metabolism. This therefore does not allow for the contribution from continuous protein, and fatty acid turnover whilst fuel intake (from the diet) is occurring. Nevertheless this is an error that could be measured by turnover studies. The particular sets of factors for both Diet O and Fasting have assumed that the metabolised amino acid composition is that of 'mammalian whole body' (Block & Weiss, 1956) - obviously not entirely correct since proline (in collagen) probably does not turnover in proportion to its large amount. The calculated fatty acid composition of triglycerides metabolised during fasting and on Diet O is also an approximation based on the composition of adipose tissue in humans on a random American diet (Field, 1965). Furthermore, it has been assumed that fatty acids are all in triglyceride form, so the proportion of glycerol will in fact be too high in its contribution to O_2 and CO_2 exchange.

An important question immediately arises concerning the use of these different factors, relating gas exchange to the composition of different fuels. Do they really make any significant quantitative difference to the calculation of fuels being metabolised? Could we not simply continue to use the 'standard' factors of Zuntz (1897), Lusk (1928) or Magnus-Levy (1907), for are these not applicable to any dietary situation?

In fact (and as shown in Table 8.2 below) it *does* appear that different amino acid patterns, and different fatty acid patterns (and to a lesser extent carbohydrate patterns since different proportions of polysaccharides lead to different eventual quantities of available glucose) do significantly affect gas exchange and thus the calculated

quantities of fat and glucose metabolised. In Table 8.2 the O_2 uptake, CO_2 output, and protein oxidation, measured continuously in an obese patient over 12 hours, are used in Equations 8.3 and 8.4 and the calculated quantities of utilised fat and glucose compared, when the constants (A, B, C, D, and E) for each diet are substituted into the equations.

TABLE 8.2. Calculated quantities of fat and glucose metabolised, using the different sets of factors (from Table 8.1). Patient E.E. on hourly intake of milk-based 'Normal' Diet A over a 12-hour period consumed 5.836 moles O_2 , produced 4.589 moles CO_2 and oxidised 24.84 g of Protein. The second group of data show the larger discrepancies that result if for the same O_2 and CO_2 values, 70.0 g Protein had been oxidised. The values are compared with those derived from Zuntz (1897).

DIET FACTORS	GLUCOSE UTILISED		FAT UTILISED	
	g/12 Hours	% Difference from Zuntz	g/12 Hours	% Difference from Zuntz
(A) PROTEIN OXIDATION 24.84 g				
Normal Diet A	34.21	14 %	42.41	6 %
Normal Diet B	33.75	12 %	42.67	6 %
Fasting	34.90	16 %	44.78	12 %
Zuntz (1897)	30.06	-	40.16	-
(B) PROTEIN OXIDATION 70.0 g				
Normal Diet A	10.14	11 %	28.01	6 %
Normal Diet B	9.58	16 %	28.40	8 %
Fasting	16.05	41 %	33.10	26 %
Zuntz (1897)	11.42	-	26.32	-

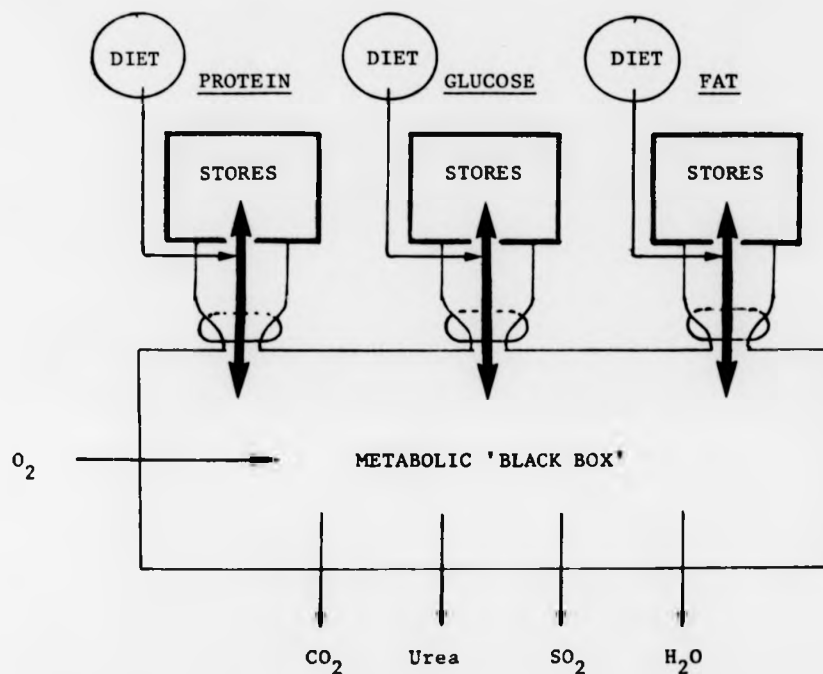
From these data it can clearly be seen that substantial differences do occur as a result of using different sets of factors for the same gas exchange data, and it can be concluded that significant error may occur if the amino acid, fatty acid, and carbohydrate composition of fuels being metabolised are not accounted for. Even if the factors themselves are marginally incorrect because of assumptions previously discussed, it would appear that they should not be ignored, in the calculation of fuel metabolism. Indeed it would seem to be a worthwhile future undertaking to derive as accurately as possible sets of such factors.

Having thus established the general formulae (Equations 8.3 and 8.4), and their required constants (Table 8.1), it was exhilarating to find that completely identical formulae could be independently derived by a 'balance' method, particularly as this approach also specifically included fuel synthesis as well as oxidation.

8.3.2. Derivation II

The model used in this approach is a stochastic one, with similar assumptions to the stochastic analysis described for whole-body protein turnover (Section 1.2 and 1.4). It is used to derive formulae expressing both oxygen-balance and carbon-balance. Figure 8.1 attempts to express the model diagrammatically (even though it may look like a multipurpose sanitation unit, or a Massey-Fergusson grain crusher).

FIGURE 8.1. A model for the derivation of formulae relating oxygen (O) and carbon (C) to fuel synthesis, interconversion, and oxidation.



The ringed isthmus () indicate the site of fuel transfer calculated by the formulae.

For this model, it is irrelevant what metabolic reactions occur within the 'black box'. It is assumed that this metabolic pool of C and O does not expand or contract, and that pathways into and out of the metabolic pool are the only important features to be considered. Diet and fuel stores and O_2 are the only significant contributions of O and C into the black box, and the only significant pathways out are body stores (and hence net synthesis of fuels), CO_2 , urea, SO_2 , and

H₂O. Small errors will be introduced by ignoring other minor pathways of oxygen and carbon in and out of the 'black box'. Thus all that matters are any net transfers of O and C in or out of the 'black box'.

From this model for O and C balance, formulae can be developed showing *net transfer of fuel into the metabolic 'black box' from diet or stores* (\equiv NET UTILISATION), and *net transfer of fuel out of the 'black box' into stores* (\equiv NET SYNTHESIS). In mathematical terms for this model, net synthesis of a fuel is simply negative net utilisation (ie. net transfer of fuel into the pool is designated +ve, and net transfer of fuel back into stores is -ve).

The term fuel 'utilisation' is deliberately used because the formulae detect net transfer in and out of the pool, but do not distinguish how a fuel is subsequently proportioned into complete oxidation and conversion to another fuel. (Neither can the (1-¹⁴C)leucine method, nor urinary-N excretion detect whether an amino acid skeleton is completely oxidised or converted into fatty acids.) Thus the formulae are derived so as to detect net transfer at the sites of the ringed isthmi on the model diagram.

Formulae are derived below, using the molar relationships from amino acid and fatty acid patterns shown for the milk-based diets (see Appendices 5.1 and 5.2),

ie. For Milk-Based Diet

<u>'Mean Amino Acid'</u>	(N)	$C_{3.825}H_{7.486}N_1O_{1.908}S_{0.031}$
<u>Glucose</u>	(C)	$C_6H_{12}O_6$
<u>'Mean Triglyceride'</u>	(F)	$C_{13.3}H_{26.55}O_{2.25}$

TABLE 8.3.

(i) O-BALANCE

<u>Moles O: IN</u> (Source)	<u>Moles O: OUT</u> (Product)
N moles x 1.908 (Protein)	N/2 x 1 (Urea)
C moles x 6 (Glucose)	0.031 N moles x 3 (SO ₂)
F moles x 2.25 (Fat)	$\frac{1}{2}(5.486)N$ moles x 1 (H ₂ O)
O ₂ moles x 2 (O ₂)	C moles x 6 (H ₂ O glucose)
	F moles x 13.28 (H ₂ O fat)
	CO ₂ moles x 2 (CO ₂ moles)
<u>BALANCE</u> 2 (O ₂ moles)	2 (CO ₂ moles) + 1.428N + 11.03F

(ii) C-BALANCE

<u>Moles C: IN</u> (Source)	<u>Moles C: OUT</u> (Product)
N moles x 3.825 (Protein)	N/2 x 1 (Urea)
C moles x 6 (Glucose)	CO ₂ moles (CO ₂)
F moles x 13.3 (Fat)	
<u>BALANCE</u> 3.325N + 6C + 13.3F	CO ₂ moles

From these balance data, 2 simultaneous equations emerge:

$$2(\text{O}_2 \text{ moles}) = 2(\text{CO}_2 \text{ moles}) + 1.428\text{N} + 11.03\text{F}$$

$$(\text{CO}_2 \text{ moles}) = 3.325\text{N} + 6\text{C} + 13.3\text{F}$$

Solution of these equations, including the conversion of N ($\times 14 \times 6.38$) to g of milk Protein (P), and use of the molecular weights of Glucose (180.18) and 'Mean Triglyceride' (222.51) for conversion of C and F to grams, incredibly leads to the following 2 formulae:

$$\text{g Glucose (C)} = 102.483(\text{CO}_2 - 0.707 \text{O}_2 - 0.0052\text{P})$$

$$\text{g Fat (F)} = 40.365(\text{O}_2 - \text{CO}_2 - 0.0079\text{P})$$

These are identical to Equations 8.3 and 8.4 with the milk-based diet factors substituted. In a similar manner, the model also gives the expected equivalent equations under the other dietary situations.

This achievement gives some confidence in the validity of the Equations 8.3 and 8.4 and their sets of factors for different fuels being metabolised. However the exciting significance of the model is that it was deliberately designed to allow for net transfer of fuel in either direction, so that net synthesis of a fuel is simply equivalent to negative net utilisation, and the equations derived, using this stochastic 'balance' principle, are identical with the previously derived equations.

Therefore the appearance of any negative values for glucose or fat utilisation, calculated from Equations 8.3 and 8.4, can confidently be equated with net synthesis of the fuel (i.e. the balance between

simultaneous total utilisation of the fuel from diet and/or stores, and total synthesis, with the balance showing a net synthesis, and thus deposition of fuel into stores).

8.3.3. Testing

As a further test of the meaning and validity of the formulae they were theoretically tested under many different hypothetical metabolic conditions. This was done by proposing different patterns of fuel oxidation, interconversion and synthesis, working out by stoichiometry the net gas exchange and net fuel transfers, and then by substituting the O_2 and CO_2 values into the formulae (which were programmed) calculating what net transfers of glucose and fat had occurred.

In all cases the calculated values of glucose and fat net utilisation or synthesis agreed entirely with the stoichiometric quantities. These included attempts to devise situations which might have the same R.Q., and complex patterns of combined oxidation, synthesis and interconversion of fuels. The formulae appeared to be able to cope with all fuel transfer situations, and it appeared impossible to have the same R.Q. produced by 2 different situations.

Two such tests, showing both stoichiometric results and the corresponding results calculated by the formulae are shown below.

1. Can 2 metabolic situations exist with the same R.Q.?

eg.

- | | | | | |
|--------------------------------------|--------|--|------------|--|
| (a) | 100 g | Glucose \rightarrow Fat | R.Q. > 1 | } In quantities so that
combined R.Q. = 1 |
| | 50 g | Glucose \rightarrow Complete oxidation | R.Q. = 1 | |
| | 39.4 g | Fat oxidation | R.Q. < 1 | |
| (b) Glucose oxidation alone R.Q. = 1 | | | | |

For the stoichiometric calculations:

- the intake was assumed to come from the milk-based diet;
- the intake of Fat was adjusted so that the combined metabolism produced an R.Q. = 1.

<u>FUEL METABOLISM</u>	<u>STOICHIOMETRY</u>	
	<u>O₂ UPTAKE</u> (moles)	<u>CO₂ OUTPUT</u> (moles)
100 g Glucose → Fat	-	0.975
50 g Glucose → Complete oxidation	1.665	1.665
39.4 g Fat → Complete oxidation	3.330	2.355
<u>TOTAL</u>	4.995 moles O ₂	4.995 moles CO ₂

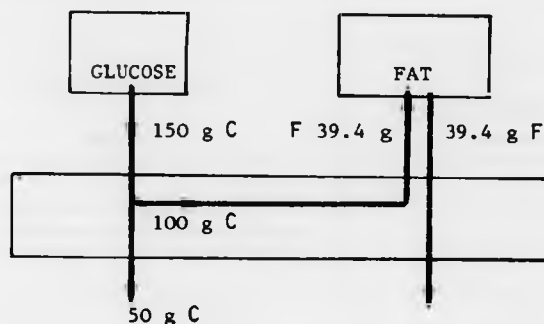
When these values for O₂ and CO₂ are substituted into the programme for the formulae (with milk-based 'Normal' Diet constants), the following results are obtained.

<u>GLUCOSE UTILISED</u>	<u>FAT UTILISED</u>
150.0 g	0 g

The formulae have, thus, verified actual net fuel metabolism.

An R.Q. = 1 is only achieved in this situation when the fat synthesised from glucose is exactly equal to fat oxidation ie. no net utilisation or synthesis of fat. The result is thus equivalent to net utilisation of 150 g glucose. This is shown in Figure 8.2, in which C and F stand for glucose and fat.

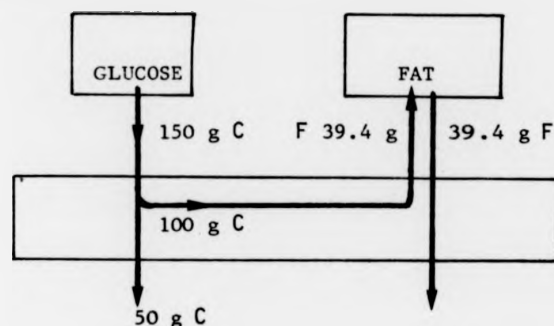
FIGURE 8.2.



2. A complex hypothetical pattern of synthesis and deposition and oxidation.

For the stoichiometric calculations, the intake was again assumed to be milk-based 'Normal' Diet A. The full stoichiometric equations are given in Appendix 5.4. The following table (Table 8.4) sets out a hypothetical *metabolic tantrum*, with fuels simultaneously being oxidised, synthesised, inter-converted and deposited in planned quantities. The stoichiometry has been calculated and the net transfers set out as 'Fuel Balance'. The ultimate net O_2 and CO_2 quantities are then substituted into the fuel utilisation formulae, along with the quantity of protein utilised.

FIGURE 8.2.



2. A complex hypothetical pattern of synthesis and deposition and oxidation.

For the stoichiometric calculations, the intake was again assumed to be milk-based 'Normal' Diet A. The full stoichiometric equations are given in Appendix 5.4. The following table (Table 8.4) sets out a hypothetical *metabolic tantrum*, with fuels simultaneously being oxidised, synthesised, inter-converted and deposited in planned quantities. The stoichiometry has been calculated and the net transfers set out as 'Fuel Balance'. The ultimate net O_2 and CO_2 quantities are then substituted into the fuel utilisation formulae, along with the quantity of protein utilised.

TABLE 8.4. Stoichiometry Balance Sheet of the 'metabolic tantrum'.

<u>FUEL METABOLISM</u> (moles)	<u>O₂ & CO₂ STOICHIOMETRY</u>	
	<u>O₂ Uptake</u> (moles)	<u>CO₂ Output</u> (moles)
10 Glucose → oxidised	60	60
10 Glucose → Glycogen stores	-	-
50 Glucose → - 15.95 Fat	-	87.9
10 AA → Oxidised	40.4	33.3
5 AA → 2.8 Glucose	3.6	-
100 AA → - 17.7 Fat	71.4	97.4
5 Fat → Oxidised	94.1	66.5
100 Fat → Fat Stores	-	-
<u>FUEL BALANCE (moles)</u>	<u>TOTAL</u>	
57.2 Glucose utilised	269.4	345.1
115.0 'Amino Acid' utilised	moles O ₂	moles CO ₂
-28.65 Fat utilised (ie. synthesised)		

When the total O₂ and CO₂ values for this complex metabolic tangle are substituted into the formulae, and the quantity of oxidised protein (115 moles 'AA' = 11,388.8 g 'AA') is also included, then the formulae indicate the following net transfers of glucose and fat.

TABLE 8.5. Glucose and Fat utilisation, comparing the results calculated by stoichiometry, with those calculated by the fuel utilisation formulae (Equations 8.3 and 8.4) with milk-based fuel factors.

	<u>From STOICHIOMETRY</u> g of Fuel	<u>BY THE FORMULAE</u> g of Fuel
GLUCOSE	10,305.2 g (57.2 moles)	10,300.4 g (utilised) (57.2 moles)
FAT	- 6,374.9 g (-28.65 g)	-6,369.2 g (synthesis) (-28.6 moles)

The excellent comparability of these results clearly indicates the potential of this formula to cope with the most complicated metabolic situations. It appears to be able to handle fasting and feeding conditions, and is able to work out net whole-body fuel transfers even when the metabolic scene is complicated by simultaneous processes of oxidation, synthesis, interconversion and deposition of different fuels. What it cannot distinguish however, are different metabolic processes occurring in different parts of the body, eg. if there were net glucose deposition in liver and net utilisation in muscle. Its measurements, like those of whole body protein turnover, reflect the net result of fuel metabolism from tissues all over the body.

Before presenting the results, it is worth summarising the contribution that this section has attempted to make so far. The principles behind the development of the formulae, and even the general formulae themselves (Equations 8.3 and 8.4), are certainly not new, being a reflection of classical indirect respirometry. What is significant however, is:

- (a) the theoretical demonstration that indirect respirometry is valid, even when synthesis and interconversion of fuels are predominant, or even with R.Q. values over 1. This is not widely accepted at present (eg. McGilvery, 1979);
- (b) that the formulae for utilisation of glucose and fat can be derived not only from the 'sum of O_2 uptakes and CO_2 outputs' method as used by Weir (1949), but also from a stochastic balance model which specifically allows for synthesis and interconversion of fuels, net synthesis being equivalent to negative net utilisation;

(c) that the amino acid, fatty acid, and carbohydrate compositions of fuels being metabolised do significantly, (and measurably) affect gas exchange. It is not sufficient to use average gas exchange values for 'protein', 'fat', and 'carbohydrate', as is usually done, using values of Zuntz (1897) or Magnus-Levy (1907), particularly if acute measurements of fuel utilisation are to be made;

(d) that glucose and fat utilisation or synthesis can be meaningfully measured acutely (eg. hourly), provided acute measurements of protein oxidation can be made. Because of slow urea pool turnover (and hence delay), the N-excretion method (even if corrected for plasma urea-pool changes) is unable to measure protein oxidation more acutely than 12-hourly. Use of an alternative method (eg. by (1-¹⁴C)leucine) can give hourly rates of protein oxidation, and these, coupled with continuous measurements of O₂ uptake and CO₂ output, enable the hourly estimation of whole-body net fuel utilisation and synthesis.

These fuel utilisation formulae therefore offer an exciting opportunity to explore whole-body energy metabolism under many widely varied conditions, not only in normal or obese subjects, but also in surgical and infected patients, and in other conditions of pathology or recovery. Three facilities are required for the success of these formulae and these include:

1. A method for acute measurement of protein oxidation (eg. (1-¹⁴C)leucine rather than N-excretion).

2. A method for accurate continuous measurement of O_2 uptake and CO_2 output (eg. a ventilated system rather than Douglas bags).
3. 'Fuel factors', as shown in Table 8.1, reflecting amino acid, fatty acid, and carbohydrate composition of the fuels being metabolised.

Whereas the first two facilities are presently available, sets of 'fuel factors', for substitution into the formulae, are not. Yet they would appear to be well worth future development, because of the potential the formulae offer for the understanding and even management of energy metabolism, in normal, pathological and recovery states.

8.4. RESULTS AND THEIR ANALYSIS

Simultaneous continuous measurements of O_2 uptake, CO_2 output, and protein oxidation by (1- ^{14}C)leucine infusion, were made in many of the subjects of the protein turnover groups shown in Section 2. This allowed the estimation of protein, carbohydrate and fat utilisation continuously over 24 - 36 hours, in the groups of subjects previously distinguished in Section 2. The results that follow show an enthralling and somewhat unexpected pattern of energy metabolism for both Control Group subjects (5 non-obese Departmental colleagues), and obese subjects (9 obese patients of the Diurnal and Diet O-Diurnal groups).

Most of the results are presented in terms of fuel utilisation over a 12-hour feeding or fasting period, as these were the periods of constant energy intake. However, it should be pointed out that these are not derived values from only a few hours measurement, but usually total 12-hour measurements. Using hourly measurements of O_2 , CO_2 and protein oxidation in the fuel-utilisation formulae, hourly rates of glucose and fat utilisation or synthesis were calculated, so that the 12-hour and 24-hour values presented in this section are actual total rates. For the protein oxidation term, it was necessary to estimate the few hourly values between feeding phase and fasting phase plateaux. This is closer to reality than assuming a constant plateau value of protein oxidation over the entire 12 hours of feeding or fasting.

When the fuel utilisation results from both Control and obese groups were calculated, it became quite clear that the pattern of fuel utilisation was particularly related to the proportion of energy intake derived from carbohydrate.

Although both obese and Control subjects were on Normal Diets A or B, there were 3 distinct intake patterns of carbohydrate within these diets. Since the protein content always remained at much the same level (at about 67 g Protein in 12 hours), carbohydrate and fat tended to have an inverse proportional relationship in these 3 intake patterns. The actual intakes of protein, carbohydrate, and fat are shown with the results further below, but Table 8.6 shows the 3 dietary patterns, with intakes expressed as a proportion of total 24-hour energy expenditure.

TABLE 8.6. Three patterns of dietary intake, with protein, carbohydrate, fat, and energy expressed as proportions of 24-hour energy expenditure. The results are means of percentages calculated for each subject (Control and obese).

INTAKE PATTERN	PERCENTAGE OF 24-HOUR ENERGY EXPENDITURE			
	PROTEIN	CARBOHYDRATE	FAT	ENERGY INTAKE
I Very low CHO (15%)	18 %	15 %	54 %	87 %
II Low CHO (30%)	16 %	30 %	76 %	121 %
III Normal CHO (50%)	17 %	50 %	43 %	111 %

The results below show that it was this intake pattern of carbohydrate that appeared to have greatest influence on fuel utilisation or deposition, for both control and obese groups. Although division of the subjects by this carbohydrate intake pattern only leaves small numbers in each group, the resultant fuel utilisation patterns are consistent and occur in both control and obese patients.

It should also be reiterated that intake of these fuels was always over a 12-hour feeding period. Because the subjects were approximately in 24-hour energy balance (87% - 121%) the feeding 12-hours was always a period of excess intake and fuel deposition, and the fasting 12-hours necessarily a period of fuel mobilisation. However the patterns of deposition and mobilisation, and the way these are apparently influenced by carbohydrate intake, are intriguing.

8.4.1. The Control Group

For each of the 3 intake patterns, the following tables show how fuel utilisation is influenced, and how the body responds in terms of deposition or mobilisation of fuels from fuel stores in each 12-hour period, as well as over 24 hours.

Group I: The Very Low Carbohydrate group

TABLE 8.7. Very Low Carbohydrate (15%) group (I), showing fuel intake, net utilisation (or synthesis = -ve values and in italics), and fuel balance in terms of deposition (shown as -ve and in italics) into stores or mobilisation from stores. (2 Subjects.)

	<u>PROTEIN</u> g Fuel per 12 (or 24) Hours	<u>CARBOHYDRATE</u>	<u>FAT</u>	<u>ENERGY</u> Kcal
<u>A. INTAKE</u>				
Feeding 12 hours	65.6	62.3	92.8	1326.7
<u>B. UTILISATION</u>				
Feeding 12 hours	43.7	159.9	- 2.4	767.6
Fasting 12 hours	25.8	127.1	6.6	648.1
<u>C. BALANCE</u>				
Feeding 12 hours	-21.9 <i>Deposited</i>	97.6 mobilised	-95.2 <i>Deposited</i>	-559.1 <i>Deposited</i>
Fasting 12 hours	25.8 mobilised	127.1 mobilised	6.6 mobilised	648.1 mobilised
Total 24 hours	3.8 mobilised	224.7 mobilised	-88.7 <i>Deposited</i>	88.8 mobilised

These results are most unexpected. Even though so little carbohydrate is supplied in the diet (62.3 g), the body appears to insist on using carbohydrate as its main fuel (287 g in 24 hours), and consequently has to withdraw large quantities from glycogen stores in both feeding and fasting periods. Furthermore, despite the large fat intake, only a minute quantity (6.6 g) is mobilised during the Fasting Phase, so that almost the entire fat intake remains stored. Some of the glycogen mobilised during the Feeding Phase, is even used for fat synthesis, this fat then adding to the fat intake deposited into fat stores.

So for this Very Low Carbohydrate intake group, 80% of Feeding Phase energy expenditure and 75% of Fasting Phase energy expenditure has come from carbohydrate, and protein oxidation has supplied most of the rest. The following table (Table 8.7) shows the proportions of energy expenditure in each phase, coming from each fuel.

TABLE 8.8. Very Low Carbohydrate group (I). Percentages of energy expenditure in each phase, derived from each fuel.

PHASE	PROTEIN	CARBOHYDRATE	FAT
Feeding 12 Hours	23.1 %	79.8 %	-2.9 %
Fasting 12 Hours	16.1 %	75.3 %	8.7 %
Total 24 Hours	20.0 %	77.7 %	2.4 %

Group II: The Low Carbohydrate Group

When the proportion of carbohydrate in the diet is increased to a level equivalent to almost 30% of total energy expenditure (= 23% of total energy intake), there is a similar pattern of fuel utilisation to the Very Low Carbohydrate group, but to a lesser extent. This is shown in the following table.

TABLE 8.9. Low Carbohydrate (28%) group (II), showing fuel intake, net utilisation or synthesis (-ve, italics), and fuel balance indicating fuel mobilisation from stores or deposition (-ve, italics) into stores. (2 Subjects)

	<u>PROTEIN</u>	<u>CARBOHYDRATE</u>	<u>FAT</u>	<u>ENERGY</u>
	g Fuel per 12 (or 24) Hours			Kcal
<u>A. INTAKE</u>				
Feeding 12 Hours	69.0	130.7	157.9	2176.5
<u>B. UTILISATION</u>				
Feeding 12 Hours	34.1	138.1	35.7	981.9
Fasting 12 Hours	19.8	109.1	36.3	817.9
<u>C. BALANCE</u>				
Feeding 12 Hours	-34.9 <i>Deposited</i>	7.4 mobilised	-122.2 <i>Deposited</i>	-1194.6 <i>Deposited</i>
Fasting 12 Hours	19.8 mobilised	109.1 mobilised	36.3 mobilised	817.9 mobilised
Total 24 Hours	-15.1 <i>Deposited</i>	116.5 mobilised	- 85.9 <i>Deposited</i>	-376.7 <i>Deposited</i>

Here is the spectacle of the body mobilising 116.5 g of glucose (worth 445 Kcal) from glycogen stores, when the diet is already supplying an excess of almost 400 Kcal. It appears not to want so much fat, at least not for immediate energy needs. So once again, the body appears to insist on using much more carbohydrate (twice as much) than the diet is offering for the 24 hour period, although to a lesser extent than was found for the Very Low Carbohydrate group (where 4.6 x the quantity supplied was used). In absolute terms, protein oxidation has remained much the same as in Group I, but while the quantity of carbohydrate utilised is less (247 g in 24 hours), fat oxidation plays a more substantial role (72 g fat in 24 hours).

So for this Low Carbohydrate group, carbohydrate oxidation is responsible for about half the energy expenditure in both Feeding and Fasting Phases.

TABLE 8.10. Low Carbohydrate (30%) group (II). Percentages of energy expenditure derived from each fuel, in each phase.

PHASE	PROTEIN	CARBOHYDRATE	FAT
Feeding 12 Hours	14.1 %	53.8 %	32.1 %
Fasting 12 Hours	9.8 %	51.0 %	39.2 %
Total 24 Hours	12.2 %	52.5 %	35.4 %

Group III: The Normal Carbohydrate Group

One of the Control Group subjects (J.K.G.) was on a much more normal diet, with carbohydrate contributing 42% of total energy intake (this amount of carbohydrate was equivalent to 47.4% of his 24 hour energy expenditure). Consequently his fuel metabolism seemed to behave much more sensibly, depositing the excess of all 3 fuels during the Feeding Phase, and mobilising them again during fasting. This pattern is shown in Table 8.11.

TABLE 8.11. Normal Carbohydrate (47%) group (III), showing fuel intake, net utilisation or synthesis, and fuel balance indicating fuel mobilisation from stores, or deposition into stores. (J.K.G.)

	<u>PROTEIN</u> g Fuel per 12 (or 24) Hours	<u>CARBOHYDRATE</u> g Fuel per 12 (or 24) Hours	<u>FAT</u> g Fuel per 12 (or 24) Hours	<u>ENERGY</u> Kcal
<u>A. INTAKE</u>				
Feeding 12 Hours	66.7	219.1	98.1	1976.9
<u>B. UTILISATION</u>				
Feeding 12 Hours	37.2	128.1	37.4	971.4
Fasting 12 Hours	23.1	73.1	47.9	796.9
<u>C. BALANCE</u>				
Feeding 12 Hours	-29.5 <i>Deposited</i>	-91.0 <i>Deposited</i>	-60.8 <i>Deposited</i>	-1005.5 <i>Deposited</i>
Fasting 12 Hours	23.1 <i>mobilised</i>	73.1 <i>mobilised</i>	47.9 <i>mobilised</i>	796.9 <i>mobilised</i>
Total 24 Hours	- 6.4 <i>Deposited</i>	-17.9 <i>Deposited</i>	-12.9 <i>Deposited</i>	-208.6 <i>Deposited</i>

This is a much more rational profile of fuel metabolism than for the previous 2 groups. Much less glucose is oxidised (201 g in 24 hours) than in the other 2 groups, so that the excess 17.9 g are still left deposited in glycogen stores after 24 hours. Fat contributes 85.3 g to energy expenditure in 24 hours - a very different situation from its contribution in Group I (where only 6.6 g fat were oxidised over 24 hours, and even then 2.4 g of fat were synthesised).

The proportional contribution of each of the fuels to energy expenditure also looks much more 'normal' as a result of this higher intake of carbohydrate in the diet. Carbohydrate and fat elegantly alternate as the main contributors to energy expenditure for Feeding and Fasting Phases. Over the 24 hour period, their proportional contribution to energy expenditure is equivalent to their proportional contribution to energy intake (42.4% for carbohydrate, 43.9% for fat).

TABLE 8.12. Normal Carbohydrate (47%) group (III). Percentages of energy expenditure derived from each fuel, in each phase.

PHASE	PROTEIN	CARBOHYDRATE	FAT
Feeding 12 Hours	15.6 %	50.4 %	34.0 %
Fasting 12 Hours	11.8 %	35.1 %	53.1 %
Total 24 Hours	13.9 %	43.5 %	42.6 %

The Control group has thus shown a remarkable and quite ironic response of whole-body fuel metabolism to the proportion of carbohydrate in adequate protein and energy containing diets. It is apparent that the less carbohydrate supplied, the more the body insists on using it as a fuel source, even if this means mobilising large quantities of glycogen, and irrespective of whether 24 hour energy intake is well in excess of 24 hour energy expenditure. Perhaps there is a distinct range, with upper and lower limits, defining the proportional contribution of glucose to total energy expenditure. The results from this group suggest glucose must contribute between 30% to 80% of energy expenditure.

These data provoke many questions in terms of both validity and significance.

- Are they an artefact due to the small group numbers? It stretches credulity somewhat to see the body utilising more and more glucose, the less the dietary intake supplies.
- If genuine, what has really triggered off such a mobilisation of glucose when the intake of glucose is very low?
- What happens when liver and muscle glycogen stores run out? The stores certainly cannot keep on donating 100 - 200 g of glucose every 24 hours.
- What happens in obese patients, having much larger fat stores?

These questions and others were at least approached, by making the same measurements in the obese patients, not only when subjected to the same carbohydrate intake categories as the Control group, but also after 3 weeks on Diet O, when it might well be expected that all glycogen stores would be completely exhausted.

8.4.2. Obese Patients

The results for the 9 obese patients, are shown in Tables 8.13 - 8.16 grouped together on the following 4 pages. Essentially they show the same tendency as seen in the Control group patients, although they also appear to have a greater ability to mobilise and use fat.

In Group I (Table 8.13) where the very low carbohydrate intake was only 17% of energy intake, equivalent to 14.4% of energy expenditure, during the feeding 12-hours when energy intake was more than 500 Kcal in excess of expenditure, the body still withdrew glucose from glycogen stores, whilst depositing protein, fat, and energy.

However, a clear difference between the obese patients and Control subjects in their response to such a low carbohydrate intake, is the ability of the obese to utilise fat. Comparison of Table 8.7 with Table 8.13 shows that whereas both groups have almost identical intakes of carbohydrate and fat, the obese group utilise nearly 50% and store nearly 50% of their fat intake during the feeding 12 hours, then during the fasting 12 hours withdraw the deposited fat - thus arriving at fat-balance after 24 hours. In complete contrast, the Control group subjects deposit their entire fat intake during the feeding 12-hours (even though this means having to withdraw large quantities of glycogen), but then appear unable to withdraw the deposited fat during the fasting 12 hours. Table 8.16 shows the percentage contribution to energy expenditure of each of the fuels, and this again reinforces the difference between obese and Control subjects (Table 8.8), in the ability of the obese to utilise fat, both from diet and fat stores, under these circumstances.

TABLE 8.13. Very Low Carbohydrate (14.4%) group (I), showing fuel intake, net utilisation or synthesis (synthesis -ve, italics), and fuel balance indicating mobilisation from stores (+ve), or deposition into stores (-ve). (5 Obese Patients.)

	<u>PROTEIN</u> g Fuel per 12 (or 24) Hours	<u>CARBOHYDRATE</u> g Fuel per 12 (or 24) Hours	<u>FAT</u> g Fuel per 12 (or 24) Hours	<u>ENERGY</u> Kcal
<u>A. INTAKE</u>				
Feeding 12 Hours	74.5	62.8	97.5	1405.0
<u>B. UTILISATION</u>				
Feeding 12 Hours	46.1	75.3	48.2	901.5
Fasting 12 Hours	24.2	61.1	50.7	779.9
<u>C. BALANCE</u>				
Feeding 12 Hours	-28.4 <i>Deposited</i>	12.5 mobilised	-49.3 <i>Deposited</i>	-503.5 <i>Deposited</i>
Fasting 12 Hours	24.2 mobilised	61.6 mobilised	50.7 mobilised	779.9 mobilised
Total 24 Hours	- 4.2 <i>Deposited</i>	73.6 mobilised	1.4 mobilised	276.4 mobilised

TABLE 8.14. Low Carbohydrate (30.5%) group (II), showing fuel intake, net utilisation or synthesis (-ve), and fuel balance indicating mobilisation from stores (+ve) or deposition into stores (-ve). (2 Obese Patients.)

	<u>PROTEIN</u> g Fuel per 12 (or 24) Hours	<u>CARBOHYDRATE</u>	<u>FAT</u>	<u>ENERGY</u> Kcal
<u>A. INTAKE</u>				
Feeding 12 Hours	69.6	131.5	140.6	2028.5
<u>B. UTILISATION</u>				
Feeding 12 Hours	36.5	102.9	36.5	864.5
Fasting 12 Hours	24.3	95.4	36.9	789.1
<u>C. BALANCE</u>				
Feeding 12 Hours	-33.2 <i>Deposited</i>	-28.6 <i>Deposited</i>	-104.1 <i>Deposited</i>	-1164.0 <i>Deposited</i>
Fasting 12 Hours	24.3 <i>mobilised</i>	95.4 <i>mobilised</i>	36.9 <i>mobilised</i>	789.1 <i>mobilised</i>
Total 24 Hours	- 8.9 <i>Deposited</i>	66.8 <i>mobilised</i>	- 67.2 <i>Deposited</i>	- 374.9 <i>Deposited</i>

TABLE 8.15. Normal Carbohydrate (52.1%) group (III), showing fuel intake, net utilisation or synthesis (-ve), and fuel balance indicating mobilisation from stores (+ve) or deposition into stores (-ve). (2 Obese Patients.)

	<u>PROTEIN</u> g Fuel per 12 (or 24) Hours	<u>CARBOHYDRATE</u>	<u>FAT</u>	<u>ENERGY</u> Kcal
<u>A. INTAKE</u>				
Feeding 12 Hours	70.4	216.2	71.8	1747.4
<u>B. UTILISATION</u>				
Feeding 12 Hours	38.3	95.5	34.2	822.4
Fasting 12 Hours	23.7	57.8	50.9	767.2
<u>C. BALANCE</u>				
Feeding 12 Hours	-32.1 <i>Deposited</i>	-120.8 <i>Deposited</i>	-37.6 <i>Deposited</i>	-925.0 <i>Deposited</i>
Fasting 12 Hours	23.7 <i>mobilised</i>	57.8 <i>mobilised</i>	50.9 <i>mobilised</i>	767.2 <i>mobilised</i>
Total 24 Hours	- 8.4 <i>Deposited</i>	- 63.1 <i>Deposited</i>	13.3 <i>mobilised</i>	-157.9 <i>Deposited</i>

TABLE 8.16. Percentages of energy expenditure derived from each fuel, and shown for each group of Groups I, II, and III.

Group I: Very Low Carbohydrate (14.4%) group.

	PROTEIN	CARBOHYDRATE	FAT
Feeding 12 Hours	20.8 %	31.9 %	47.3 %
Fasting 12 Hours	12.6 %	30.0 %	57.4 %
Total 24 Hours	17.0 %	31.0 %	52.0 %

Group II: Low Carbohydrate (30.5%) group.

	PROTEIN	CARBOHYDRATE	FAT
Feeding 12 Hours	17.2 %	45.5 %	37.4 %
Fasting 12 Hours	12.5 %	46.3 %	41.3 %
Total 24 Hours	14.9 %	45.9 %	39.3 %

Group III: Normal Carbohydrate (52.1%) group.

	PROTEIN	CARBOHYDRATE	FAT
Feeding 12 Hours	18.9 %	44.4 %	36.7 %
Fasting 12 Hours	12.6 %	28.8 %	58.7 %
Total 24 Hours	15.8 %	36.9 %	47.3 %

With the Low Carbohydrate group (II), once again protein, carbohydrate and fat intakes are almost identical for obese (Table 8.14) and Control (Table 8.9) groups. Again both groups show the same tendency to insist on utilising much more glucose than is supplied by food intake, although for both groups the effect is not as severe as for the Very Low Carbohydrate groups, and once again the obese are less affected than Control group subjects. Thus for both Control and obese groups, despite an energy intake 375 Kcal in excess of 24 hour energy expenditure, and despite net deposition of large quantities of fat into fat stores over the 24 hours, glucose is still withdrawn in large quantities (116.5 g in Controls; 66.8 g in obese) from glycogen stores.

Table 8.16 (Group II) further demonstrates this effect, showing how carbohydrate metabolism is expected to contribute a disproportionately large 46% of energy expenditure, when it only receives 30% (of energy expenditure) as carbohydrate in the diet. For the Control group (Table 8.10) carbohydrate had to contribute a disproportionate 53% of energy expenditure, for the same 30% carbohydrate intake.

As with the Control subject, the obese patients in the Normal Carbohydrate group (III) showed a more expected profile of fuel metabolism, all fuels showing net deposition in the feeding 12 hours and net withdrawal from stores in the fasting 12 hours. It is interesting to note that both Control and obese patients have utilised almost exactly the same quantity of fat over 24 hours, but because the fat intake in the obese group (71.8 g fat) is substantially smaller than the fat intake of the Control group subject (98.1 g fat), the obese patients end up using more fat than they take in, and so in a situation parallel to low carbohydrate intakes, fat has to be withdrawn from stores and utilised, despite an energy intake in excess of 24 hour energy

expenditure, and simultaneous net deposition of glucose and protein.

This pattern is further reinforced by the energy metabolism profile of obese patient E.E. (continuous hourly intake over 24 hours). Her very high carbohydrate intake (245 g) contributed the equivalent of 76% of her energy expenditure (and 50% of her energy intake), seen in Table 8.17. Yet her energy metabolism deliberately chooses to use as little glucose as possible for energy expenditure (only 26%). Rather, it decides that fat should contribute 57% to energy expenditure - more than the diet supplies. Hence, fat mobilisation from stores is necessary, even though 160 g glucose are in excess and have to be deposited, and with energy intake 630 Kcal in excess of expenditure.

TABLE 8.17. Patient E.E., having a very high proportion of carbohydrate in the diet (= 76% energy expenditure), showing intake, utilisation, and deposition or mobilisation of fuels.

	PROTEIN	CARBOHYDRATE	FAT	ENERGY
	g Fuel per 24 Hours			Kcal
INTAKE (24 Hours)	72.3	245.4	72.2	1870.4
UTILISATION (24 Hours)	49.7	85.8	80.3	1240.1
% of Total Utilisation	16.3 %	26.4 %	57.3 %	100 %
BALANCE (24 Hours)	-22.6 <i>Deposited</i>	-159.6 <i>Deposited</i>	8.1 <i>mobilised</i>	-630.3 <i>Deposited</i>

Thus a pattern seems to emerge, both from Control and obese subjects, in which energy metabolism appears to insist on utilising *most* the fuel (glucose or fat) which is *least* available from intake. This pattern is visibly apparent in Figure 8.3, where the contrast

expenditure, and simultaneous net deposition of glucose and protein.

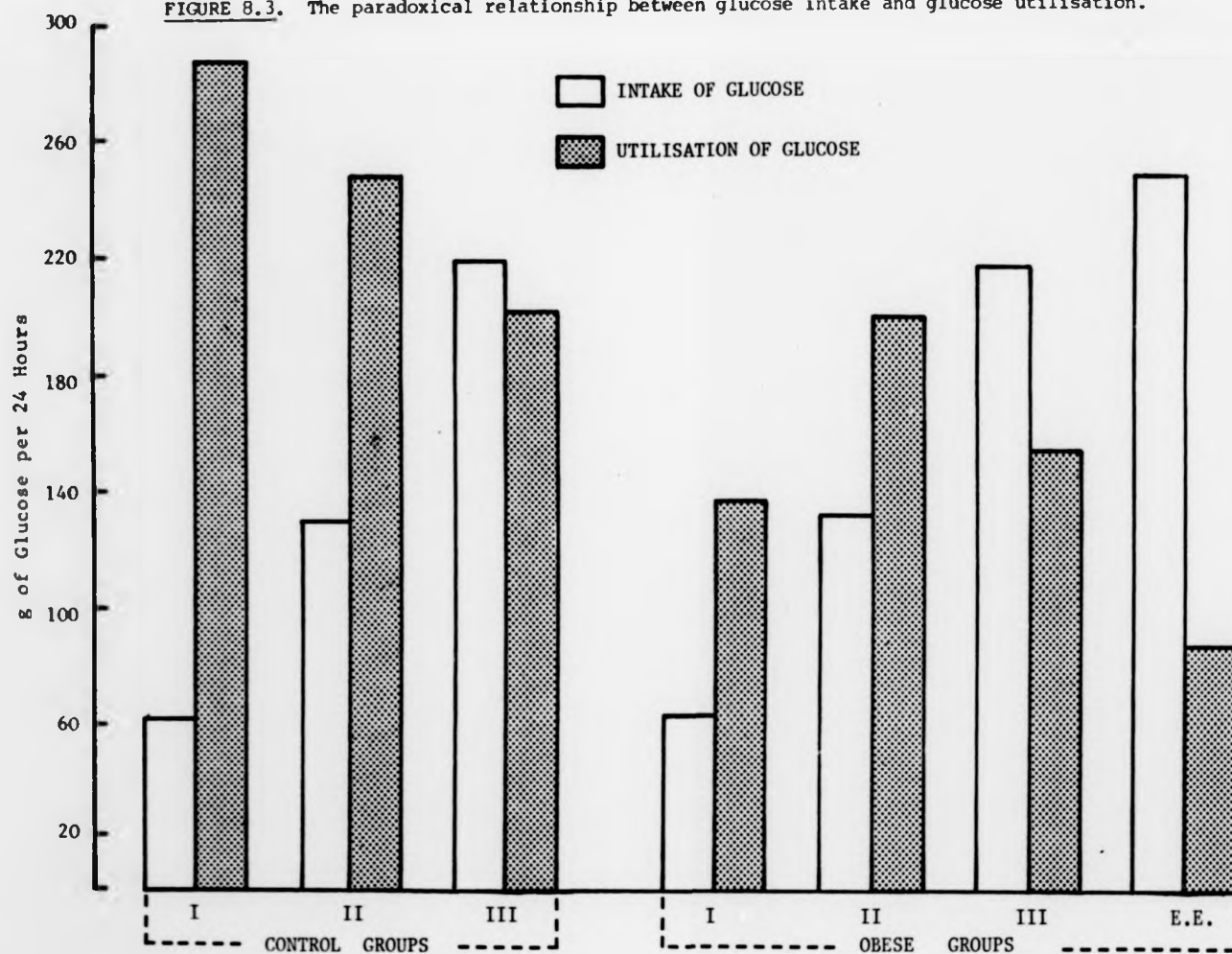
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% of Total Utilisation	16.3 %	26.4 %	57.3 %	100 %
BALANCE (24 Hours)	-22.6 <i>Deposited</i>	-159.6 <i>Deposited</i>	8.1 <i>mobilised</i>	-630.3 <i>Deposited</i>

Thus a pattern seems to emerge, both from Control and obese subjects, in which energy metabolism appears to insist on utilising *most* the fuel (glucose or fat) which is *least* available from intake. This pattern is visibly apparent in Figure 8.3, where the contrast

FIGURE 8.3. The paradoxical relationship between glucose intake and glucose utilisation.



between 24 hour glucose intake and utilisation is shown for all groups. Although the Very Low Carbohydrate group (I) of obese patients appears to show a 24 hour glucose utilisation inconsistent with the rest of the pattern in Figure 8.3, this may result from its substantial energy deficit over 24 hours. During the Feeding 12-hours this group still shows the characteristic glucose mobilisation pattern, despite excess fat and energy intake.

Bearing in mind this paradoxical pattern and the question it raises as to what energy metabolism does when glycogen supplies are exhausted, fuel metabolism was measured in the obese patients on Diet O, after 3 weeks on only 500 Kcal of glucose syrup per day, a situation in which one might expect glycogen stores to be exhausted. Table 8.18 shows how energy metabolism copes with this affront.

TABLE 8.18. Fuel intake, its net utilisation, and fuel balance in terms of mobilisation from stores or deposition (-ve) into stores, in 4 obese patients after 3 weeks on Diet O (500 Kcal/day glucose syrup).

	<u>PROTEIN</u> g Fuel per 12 (or 24) Hours	<u>CARBOHYDRATE</u>	<u>FAT</u>	<u>ENERGY</u> Kcal
<u>A. INTAKE</u>				
Feeding 12 Hours	0	129.4	0	494.7
<u>B. UTILISATION</u>				
Feeding 12 Hours	7.1	68.1	46.3	698.1
Fasting 12 Hours	6.5	49.2	45.3	615.1
<u>C. BALANCE</u>				
Feeding 12 Hours	7.1 mobilised	-61.4 <i>Deposited</i>	46.3 mobilised	203.4 mobilised
Fasting 12 Hours	6.5 mobilised	49.2 mobilised	45.3 mobilised	615.1 mobilised
Total 24 Hours	13.6 mobilised	-12.1 <i>Deposited</i>	91.6 mobilised	819.0 mobilised

These data show the amazing versatility of the body to ensure that glucose is always available to contribute substantially to energy expenditure. Feeding phase energy expenditure is almost 60 Kcal/hour, whilst the energy intake is only 41 Kcal/hour - yet still the body deposits half this intake each hour, so that a glucose reserve can accumulate. This is achieved against an energy deficit of 200 Kcal during the Feeding phase (amounting to a deficit of over 800 Kcal in the 24 hours).

So although the entire energy intake is only 129.4 g of glucose, the body only uses 68 g during the Feeding phase, depositing the remaining 61.4 g into glycogen stores, and then subsequently withdrawing most of this during the Fasting phase. It is as though the body has learnt to anticipate how much glucose it needs to store during the intake period, to maintain an adequate supply for fasting energy expenditure.

Table 8.19 shows how the proportional contribution of glucose to energy expenditure remains adequate in Feeding and Fasting phases, with fat mobilisation and oxidation supplying some 2/3 of the energy.

TABLE 8.19. The proportional contribution of fuels to energy expenditure, in obese patients having a daily intake of only 500 Kcal of glucose syrup.

PHASE	PROTEIN	CARBOHYDRATE	FAT
Feeding 12 Hours	4.1 %	37.3 %	58.6 %
Fasting 12 Hours	4.3 %	30.5 %	65.2 %
Total 24 Hours	4.2 %	34.1 %	61.7 %

The results from all these whole-body fuel metabolism measurements demonstrate a remarkable series of responses by the body to different levels of carbohydrate intake. Both non-obese and obese subjects appear to respond in the same way, although the obese do so less dramatically. Although subject numbers in each group are small, the patterns of fuel metabolism found in both obese and non-obese groups, suggest the following 3 conclusions:

1. The proportion of whole-body energy expenditure contributed from glucose seems to be inversely influenced by the proportion of carbohydrate in an incoming diet, when adequate energy and protein are supplied. Thus the closer energy intake from carbohydrate approaches energy expenditure, the less carbohydrate is actually used for energy expenditure. Conversely, the lower the intake of carbohydrate (or ? fat), the higher its proportional contribution to energy expenditure.
2. Obese subjects appear to be able to oxidise and mobilise fat more readily than non-obese subjects, at least when there is an abundance of fat but little carbohydrate in the diet.
3. It appears that the body attempts to ensure that at least a proportion of energy expenditure is derived from glucose, even when very little is supplied in the diet. In response to a prolonged daily intake of only 129 g of glucose syrup, the body not only 'adapted' its protein metabolism (see Section 6), but 'learnt' to store half this meagre intake during the feeding period, so that glucose could supply at least 30% of total energy expenditure in both feeding and fasting periods. In all the situations

measured above, glucose never supplied less than 25% of total energy expenditure (and this occurred when glucose intake was highest - equivalent to 76% of expenditure).

These phenomena are difficult to explain, and provoke many questions about their significance and the dynamic role of glucose and fat in fuel metabolism, (as well as a slight nervousness about their validity because of small group numbers and the method itself). Here indeed is much scope for further investigation.

8.5. SUMMARY AND CONCLUDING COMMENTS

It was the development of techniques for the (1-¹⁴C)leucine method, particularly those required for prolonged and diurnal measurements of whole-body protein turnover, that led to an appreciation of their potential for continuously measuring whole-body energy metabolism and changes in body fuel stores as well. The particular techniques that offered these new opportunities were those that allowed the continuous measurement of protein oxidation rates, even on an hourly basis, as well as continuous accurate measurement of O₂ uptake and CO₂ output over 24 hours.

It has been shown in this section that the principles of metabolic mixture measurement were originally developed by Zuntz, Lusk, Benedict and others, well over 50 years ago, and the basic formulae developed here in this section are really only an expression of their principles. However it was also shown that relatively little research appears to have been done on fuel utilisation patterns, one of the reasons being that the techniques currently used (N-excretion for protein oxidation; Douglas bag for O₂ and CO₂) have remained largely unchanged, and these are quite inadequate for accurate or sensitive measurement of acute changes in protein oxidation or gas exchange. The other main limiting factor seems to have been the concept that indirect calorimetry is invalid unless oxidation is predominantly occurring, restricting its use mainly to conditions of fasting or prolonged exercise. This concept is still widely held.

The contributions that this section attempts to make are both theoretical and practical. Fuel utilisation formulae were derived by the 'O₂ uptake/CO₂ output' method of Weir (1949), but then rederived from a stochastic balance model which specifically allowed for fuel

synthesis and interconversion. In this way it was shown that indirect calorimetry is theoretically valid even when synthesis, deposition, interconversion, or oxidation of fuels are occurring (both Lusk and Benedict tested and accepted this).

It was then shown that if O_2 , CO_2 and protein oxidation can be accurately measured, then the formulae for fuel utilisation require account to be taken of the actual amino acid, fatty acid, and carbohydrate composition of fuels being immediately metabolised. Appropriate sets of factors, for substitution into the fuel utilisation formulae, were calculated for each of several diets, including fasting, and it was clearly shown that gas exchange, and hence fuel utilisation patterns, were significantly and measurably affected by different amino acid and fatty acid compositions. It was suggested that general gas exchange factors for 'protein', 'fat', and 'carbohydrate' (eg. those of Zuntz, 1897) are inadequate if fuel utilisation patterns are to be investigated, and that future worthwhile work in this area might include the development of such specific factors for specific foods.

The formulae, and their appropriate factors, were then used to investigate the fuel utilisation patterns and responses in groups of obese and non-obese subjects. Their hourly protein oxidation rates, O_2 uptake and CO_2 output quantities were used in the formulae, and 12-hour patterns of feeding phase and fasting phase fuel metabolism were built up and then compared, for diets distinguished by their different proportions of carbohydrate.

The unexpected profiles that emerged seemed to indicate that the body attempts to ensure that glucose contributes to energy expenditure, that the less glucose there is in the diet, the more the body uses for energy expenditure and vice versa, and that this effect is less marked

in obese subjects because of their ability to withdraw fat from stores and oxidise it more readily than the non-obese.

This section has thus attempted to demonstrate that these fuel utilisation formulae are an exciting tool, with much wider validity than has been realised. The use of more accurate and sensitive techniques for measurement of protein oxidation, O_2 uptake and CO_2 output, and the analysis and development of appropriate factors for fuels being metabolised, accounting for their amino acid, fatty acid and carbohydrate contents, give these formulae tremendous investigative potential. Ahead, largely uncharted, lies human whole-body energy metabolism under many conditions, including health, disease, trauma, recovery and growth.

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APPENDICES

1 - 5

APPENDIX 1.1. 'DIET O' GROUP OF PATIENTS. INDIVIDUAL DIETARY and ANTHROPOMETRIC DATA

NAME	SEX	DAILY DIETARY INTAKE Kcal/Protein	Days	AGE (Yrs)	HEIGHT (cm)	INITIAL WEIGHT (Kg)	% of IDEAL WEIGHT (%)	WEIGHT LOSS Kg	'WEIGHT LOSS/DAY' g/day
DR	F	1868/72 479/ O	3 21	46	164	101.5	163.7 %	6.0	286
MK	F	1809/67 479/ O	3 20	60	161	107.0	178.3 %	5.5	275
MM	M	1603/62 479/ O	3 20	55	171	109.0	155.7 %	8.0	400
MP	F	1603/62 479/ O	3 21	48	159	107.0	184.5 %	7.5	357
ME	F	1602/62 477/ O	3 20	54	160	82.0	139 %	5.5	275
MW	F	2250/72 495/ O	3 19	59	169	106.8	161.8 %	5.5	290
TD	F	1807/67 478/ O	3 20	41	160	94.8	160.6 %	6.5	325
EK	F	1625/68 495/ O	3 19	46	165	88.4	140.3 %	7.3	384
RO	F	1870/72 495/ O	3 19	47	155	84.7	153.9 %	5.7	297
MEAN ± S.D.		1782/67 484/ O	3 20	50.7 ± 6.6	162.7 ± 5.1	97.9 ± 10.7	159.8 % ± 15	6.4 ± 0.98	321 ± 48

APPENDIX 1.2. 'DIET P' GROUP OF PATIENTS. INDIVIDUAL DIETARY and ANTHROPOMETRIC DATA

NAME	SEX	DAILY DIETARY INTAKE Kcal/Protein	Days	AGE (Yrs)	HEIGHT (cm)	INITIAL WEIGHT (Kg)	% of IDEAL WEIGHT (%)	WEIGHT LOSS Kg	'WEIGHT LOSS/DAY' g/day
MB	F	1868/72 528/48	3 20	42	175	86.5	123.0 %	4.5	225
SB	M	2361/72 531/48	3 20	54	170	175.0	231.8 %	10.0	500
MR	F	1868/72 528/48	3 20	59	155	118.0	195.0 %	6.0	300
MM	F	1603/62 456/48	3 20	44	163	108.5	177.9 %	5.5	275
MEAN		1925/70	3	49.8	165.8	122.0	181.9 %	6.5	325
± S.D.		510/48	20	± 8.1	± 8.7	± 37.7	+ 45.3	+ 2.4	+ 120

APPENDIX 1.3. Individual protein turnover rates, in μ moles leucine per hour, for 9 obese patients, measured over the 12-hour feeding phase, initially on a 'Normal Diet', and then after 3 weeks on 'Diet O'.

A. NORMAL DIET

	Q	Z	B	E	I
PATIENT	μ moles Leucine per Hour				
DR	7564.2	5792.7	3121.7	1771.5	4442.5
MK	8446.3	5137.9	4547.9	3308.4	3898.4
MM	7918.6	6695.2	4095.4	1223.4	3823.2
MP	8944.4	6552.6	5121.2	2391.7	3823.2
ME	8676.4	6418.4	4868.6	2257.6	3807.8
MW	8877.2	7137.7	4508.4	1739.5	4368.8
TD	8366.6	5953.0	4358.0	2413.6	4008.6
EK	9158.9	6813.4	4780.5	2345.5	4378.4
RO	6460.8	4587.8	2000.4	1873.0	4460.4
<u>MEAN</u>	8268.2	6121.0	4155.8	2147.1	4112.4
\pm S.D.	\pm 345	\pm 834	\pm 992	\pm 588	\pm 292

B. DIET O FOR 3 WEEKS

	Q	Z	B	E	I
PATIENT	μ moles Leucine per Hour				
DR	5217.6	4827.8	5217.6	389.7	O
MK	3684.0	3397.8	3684.0	286.2	O
MM	4917.8	4597.7	4917.8	320.1	O
MP	3633.0	3204.6	3633.0	428.4	O
ME	3744.4	3430.6	3744.4	313.8	O
MW	4165.9	3866.4	4165.9	299.5	O
TD	4492.4	4045.2	4492.4	447.2	O
EK	4190.9	3747.4	4190.9	443.5	O
RO	3245.8	2943.3	3245.8	302.5	O
<u>MEAN</u>	4143.5	3784.5	4143.5	359.0	O
\pm S.D.	\pm 643	\pm 626	\pm 643	\pm 67	

APPENDIX 1.4. Individual protein turnover rates, in g Protein per 12 Hours, for the 9 obese patients initially on 'Normal Diet' and then after 3 weeks on 'Diet O', the measurements made over the feeding 12 hours. (The t-test for matched pair samples is used to test for significant difference in Q, Z, B and E on the two diets.)

A. NORMAL DIET

	Q	Z	B	E	I
PATIENT	g Protein per 12 Hours				
DR	148.8	114.0	61.4	34.9	72.1
MK	166.2	101.1	89.5	65.1	66.9
MM	155.8	131.8	80.6	24.1	62.0
MP	176.0	128.9	100.8	47.1	62.0
ME	170.7	126.3	95.8	44.4	61.8
MW	174.7	140.5	88.7	34.2	72.3
TD	164.6	117.1	85.8	47.5	66.9
EK	180.2	134.1	94.1	46.2	68.4
RO	127.1	90.3	39.4	36.9	72.3
<u>MEAN</u>	162.7	120.4	81.8	42.3	67.2
± S.D.	± 16.6	± 16	± 19.5	± 11.6	± 4.5

B. DIET O FOR 3 WEEKS

	Q	Z	B	E	I
PATIENT	g Protein per 12 Hours				
DR	102.7	95.0	102.7	7.7	0
MK	72.5	66.9	72.5	5.6	0
MM	96.8	90.5	96.8	6.3	0
MP	71.5	63.1	71.5	8.4	0
ME	73.7	67.5	73.7	6.2	0
MW	82.0	76.1	82.0	5.9	0
TD	88.4	79.6	88.4	8.8	0
EK	82.5	73.8	82.5	8.7	0
RO	63.9	57.9	63.9	6.0	0
<u>MEAN</u>	81.5	74.5	81.5	7.0	0
± S.D.	± 12.4	± 12.3	± 12.6	± 1.3	
t-test	p < 0.001	p < 0.001	N.S.D.	p < 0.001	

APPENDIX 1.5. Individual protein turnover rates for 9 obese patients, with the rate after 3 weeks on Diet O expressed as a percentage of the rate on the Normal Diet for each patient. Measurements made over feeding 12 hours for both diets.

A. NORMAL DIET

	Q	Z	B	E	I
ALL PATIENTS	100 %	100 %	100 %	100 %	100 %

B. DIET O FOR 3 WEEKS

	Q	Z	B	E	I
PATIENT					
DR	69.0 %	83.3 %	167.3 %	22.1 %	0
MK	43.6 %	66.2 %	81.0 %	8.6 %	0
MM	62.1 %	68.7 %	120.1 %	26.1 %	0
MP	40.6 %	49.0 %	70.9 %	17.8 %	0
ME	43.2 %	53.4 %	76.9 %	14.0 %	0
MW	46.9 %	54.2 %	92.4 %	17.3 %	0
TD	53.7 %	68.0 %	103.0 %	18.5 %	0
EK	45.8 %	55.0 %	87.7 %	18.8 %	0
RO	50.3 %	64.1 %	162.2 %	16.3 %	0
MEAN	50.6 %	62.4 %	106.8 %	17.7 %	0
± S.D.	± 9.5	± 10.6	± 35.9	± 4.9	

APPENDIX 1.6. Individual protein turnover rates, in g Protein per 12 Hours, for the 4 obese patients initially on 'Normal Diet' and then after 3 weeks on 'Diet P', the measurements made over the feeding 12 hours. (The t-test for matched pair samples is used to test for significant difference in Q, Z, B, and E, on the different diets.)

A. 'NORMAL DIET' FOR 3 DAYS

	Q	Z	B	E	I
PATIENT	g Protein per 12 Hours				
MB	186.4	150.6	99.0	35.8	72.1
SB	207.2	163.2	119.2	44.0	72.0
MR	144.1	99.6	56.7	44.5	72.1
MM	167.3	126.0	92.1	41.3	62.0
MEAN	176.3	134.9	91.8	41.4	70.0
± S.D.	± 27	± 28	± 26	± 4	± 5

B. 'DIET P' FOR 3 WEEKS

	Q	Z	B	E	I
PATIENT	g Protein per 12 Hours				
MB	141.0	114.8	80.6	26.2	48.1
SB	187.5	152.2	126.9	35.3	48.3
MR	119.0	86.1	58.6	32.9	48.1
MM	130.5	107.0	69.9	23.4	48.3
MEAN	144.5	115.0	84.0	29.5	48.2
± S.D.	± 30	± 28	± 30	± 5.6	± 0.1
t-test	p < .02	.02 < p < .05	N.S.D.	p < .02	

APPENDIX 1.7. Individual protein turnover rates for 4 obese patients, the rate after 3 weeks on 'Diet P' being expressed as a percentage of the rate on 'Normal Diet' for each patient. Measurements made over the feeding 12 hours, for both diets.

A. 'NORMAL DIET' FOR 3 DAYS

	Q	Z	B	E	I
ALL PATIENTS	100 %	100 %	100 %	100 %	100 %

B. 'DIET P' FOR 3 WEEKS

	Q	Z	B	E	I
PATIENTS					
MB	75.6 %	76.2 %	81.4 %	73.2 %	66.8 %
SB	90.5 %	93.3 %	106.5 %	80.2 %	67.1 %
MR	82.6 %	86.5 %	103.4 %	73.9 %	66.8 %
MM	78.0 %	84.9 %	75.9 %	56.7 %	77.9 %
MEAN	81.7 %	85.2 %	91.8 %	71.0 %	69.7 %
± S.D.	± 6.6	± 15	± 15	± 10	± 5.5

APPENDIX 1.8 Individual protein turnover rates, in g Protein per 12 hours, for 6 obese patients initially on 'Normal' Diet and then for 3 weeks on either Diet P or Diet O. Measurement by multiple dose ¹⁵N-Glycine/NH₃ end product method.

NORMAL DIET	Q	Z	B	E	I
g Protein per 12 Hours					
M.Mc	197.9	155.6	135.9	42.3	62.0
MP	161.8	115.4	99.8	46.4	62.0
CW	220.7	187.1	158.7	33.6	62.0
M.Ma	160.9	113.9	98.9	47.0	62.0
RH	227.8	199.3	165.8	28.5	62.0
RQ	220.1	179.9	158.1	40.1	62.0
MEAN	198.2	158.5	136.2	39.7	62.0
±S.D.	±30	±37	±30	±7	

DIET P FOR 3 WEEKS

	Q	Z	B	E	I
g Protein per 12 Hours					
M.Ma	172.5	123.9	131.1	48.6	41.4
RH	215.1	205.3	166.8	9.8	48.3
RQ	194.2	174.5	145.9	19.7	48.3
MEAN	193.9	167.9	147.9	26.0	46.0
±S.D.	±21	±41	±18	±20	±4

DIET O FOR 3 WEEKS

	Q	Z	B	E	I
M.Mc	138.9	124.3	138.9	14.7	0
MP	70.9	59.5	70.9	11.4	0
CW	103.4	93.5	103.4	9.9	0
MEAN	104.4	92.4	104.4	12.0	0
±S.D.	±34	±32	±34	±2.5	0

Statistical comparison of Diet P and Diet O against 'Normal' Diet t-test

	Z	B
Diet P	N.S.D	N.S.D
Diet O	p<.05	N.S.D.

APPENDIX 2.1 'DIURNAL' and 'DIET O - DIURNAL' GROUPS of OBESE PATIENTS. DIETARY and ANTHROPOMETRIC DATA.

NAME	SEX	DAILY DIETARY INTAKE Kcal/Protein	AGE (yrs)	HEIGHT (cm)	WEIGHT (kg)	% of IDEAL WEIGHT (%)
FB	F	1516/80.9	63	161	99.0	167.8%
SH	F	1358/71.4	45	160	77.6	131.5%
DH	F	1335/69.9	56	165	81.0	128.6%
MH	F	1358/71.4	57	161	99.0	167.8%
ML	F	1494/73.8	43	161	117.0	198.3%
MT	F	1457/78.6	46	150	118.0	224.8%
MW	F	2250/72.3	59	169	106.8	161.8%
TD	F	1807/66.9	41	160	94.8	160.6%
EK	F	1625/68.4	46	165	88.4	140.3%
RO	F	1870/72.3	47	155	84.7	153.9%
MEAN		1607/72.59	50.3	160.7	96.6	163.5%
±S.D.		±291 4.3	±7.7	±5.3	±14.2	

APPENDIX 2.2. Individual protein turnover rates, in grams of protein per 12 hours, for 10 obese patients on 'Normal' Diet, measured over both Feeding 12 hours, and Fasting 12 hours ('Diurnal' and 'Diet-O Diurnal' groups).

A. FEEDING 12 HOURS

<u>NAME</u>	<u>Q</u>	<u>Z</u>	<u>B</u>	<u>E</u>	<u>I</u>
g Protein per 12 hours					
FB	176.0	120.7	77.0	55.3	80.9
SH	158.7	109.0	71.4	49.7	71.4
DH	170.8	127.2	85.4	43.6	69.9
MH	172.9	116.4	85.6	56.5	71.4
ML	151.0	107.3	66.0	43.8	73.8
MT	184.1	131.5	87.7	52.7	78.6
MW	174.7	140.5	88.7	34.2	72.3
TD	164.6	117.1	85.8	47.5	66.9
EK	180.2	134.1	94.1	46.2	68.4
RO	127.1	90.3	39.4	36.9	72.3
MEAN	166.0	119.4	78.1	46.6	72.6
±S.D.	±16.9	±14.9	±16.1	±7	±4.3

B. FASTING 12 HOURS

<u>NAME</u>	<u>Q</u>	<u>Z</u>	<u>B</u>	<u>E</u>	<u>I</u>
g Protein per 12 hours					
FB	101.0	81.9	101.0	19.1	0
SH	84.7	70.5	84.7	14.4	0
DH	108.0	90.0	108.0	18.0	0
MH	105.1	80.3	105.1	24.8	0
ML	103.7	90.3	103.7	13.4	0
MT	112.7	95.5	112.7	17.2	0
MW	150.8	125.5	150.8	25.3	0
TD	84.8	70.2	84.8	14.6	0
EK	106.7	86.7	106.7	20.0	0
RO	105.5	85.5	105.5	19.9	0
MEAN	106.3	87.6	106.3	18.7	0
±S.D.	±18.2	±15.6	±18.2	±4.1	
Matched pair t-test	p<.001	p<.001	P<.01	P<.001	

APPENDIX 2.3. Individual protein turnover rates, expressed in millimoles of Leucine per Hour, for the 10 obese patients on 'Normal' Diet ('Diurnal' and 'Diet O-Diurnal' groups), measured for diurnal changes with (1-¹⁴C)leucine infusion over 24 hours.

A. FEEDING 12 HOURS

NAME	Q	Z	B	E	I
		millimoles Leucine per Hour			
FB	9.94	6.13	3.91	2.81	5.03
SH	8.06	5.54	3.63	2.52	4.43
DH	8.68	6.47	4.34	2.21	4.34
MH	8.79	5.92	4.35	2.87	4.44
ML	7.67	5.45	3.35	2.22	4.32
MT	9.36	6.68	4.46	2.68	4.90
MW	8.88	7.14	4.51	1.74	4.37
TD	8.37	5.95	4.36	2.42	4.01
EK	9.16	6.81	4.78	2.35	4.38
RO	6.46	4.59	2.00	1.87	4.46
Mean	8.44	6.07	3.97	2.37	4.47
± S.D.	±0.86	±0.75	±0.82	±0.37	±0.29

B. FASTING 12 HOURS

NAME	Q	Z	B	E	I
		millimoles Leucine per Hour			
FB	5.13	4.16	5.13	0.97	0
SH	4.32	3.59	4.32	0.73	0
DH	5.49	4.57	5.49	0.92	0
MH	5.34	4.08	5.34	1.26	0
ML	5.27	4.59	5.27	0.68	0
MT	5.73	4.86	5.73	0.87	0
MW	7.67	6.38	7.67	1.29	0
TD	4.31	3.57	4.31	0.74	0
EK	5.42	4.41	5.42	1.01	0
RO	5.36	4.35	5.36	1.01	0
Mean	5.40	4.45	5.40	0.95	0
± S.D.	±0.93	±0.79	±0.93	±0.21	

APPENDIX 2.4. Control Group of Subjects. Dietary and Anthropometric Data

NAME	SEX	DAILY DIETARY INTAKE Kcal/Protein	AGE (yrs)	HEIGHT (cm)	WEIGHT (Kg)	% of IDEAL WEIGHT (%)
MG	F	1327/65.6	43	170	54.0	80.6 %
JS	F	1327/65.6	58	167	55.0	84.6 %
JCW	M	2103/65.8	61	170	80.0	115.0 %
RWS	M	2250/72.3	53	173	80.0	112.7 %
JKG	M	1977/66.7	67	178	66.0	88.0 %
MEAN	2F	1797/67.2	56.4	172	67.0	96.2 %
± S.D.	3M	±440/±2.9	±9	±4.2	±12.8	±16

APPENDIX 2.5 'Control' group individual protein turnover rates,
expressed in grams of protein per 12 hours, for both feeding 12 hours
and fasting 12 hours.

A. FEEDING 12 HOURS

NAME	Q	Z	B	E	I
g Protein per 12 Hours					
MG	133.2	82.5	53.3	50.7	65.6
JS	149.5	100.8	69.4	48.7	65.6
JCW	180.5	148.9	104.7	31.7	65.8
RWS	161.9	118.6	75.9	43.4	72.3
JKG	151.2	110.4	72.3	40.8	66.7
MEAN	155.3	112.2	75.1	43.1	67.2
±S.D.	±17	±24.5	±19	±7.5	±2.9

B. FASTING 12 HOURS

	Q	Z	B	E	I
g Protein per 12 Hours					
MG	75.8	59.0	75.8	16.8	O
JS	106.7	81.7	106.7	25.0	O
JCW	111.7	95.6	111.7	16.1	O
RWS	112.7	95.5	112.7	17.2	O
JKG	95.7	76.1	95.7	19.6	O
MEAN	100.5	81.6	100.5	18.9	O
±S.D.	±15	±15	±15	±4	

	Q	Z	B	E
Matched pair t-test	p<.001	p<.01	P<.01	p<.01

APPENDIX 2.6. Individual protein turnover rates for the 'Control Group' of 5 non-obese patients, expressed as millimoles of Leucine per Hour, and measured over 24 hours, using (1-¹⁴C)leucine infusion.

A. FEEDING 12 HOURS

NAME	Q	Z	B	E	I
		millimoles of Leucine per Hour			
MG	6.78	4.20	2.71	2.58	4.07
JS	7.60	5.12	3.53	2.48	4.07
JCW	9.17	7.56	5.32	1.61	3.86
RWS	8.23	6.02	3.86	2.21	4.37
JKG	7.68	5.61	3.67	2.07	4.01
Mean	7.89	5.70	3.82	2.19	4.07
± S.D.	±0.88	±1.2	±0.95	±0.38	±0.19

B. FASTING 12 HOURS

NAME	Q	Z	B	E	I
		millimoles of Leucine per Hour			
MG	3.86	3.00	3.86	0.86	0
JS	5.42	4.15	5.42	1.27	0
JCW	5.68	4.86	5.68	0.82	0
RWS	5.72	4.85	5.72	0.87	0
JKG	4.86	3.87	4.86	0.99	0
Mean	5.11	4.15	5.11	0.96	0
± S.D.	±0.78	±0.77	±0.78	±0.18	

APPENDIX 2.7. Protein and leucine balances for obese (Diurnal and Diet O-Diurnal groups) and control subjects.

Leucine excretion is calculated at dietary protein proportion until balance, and whole body protein proportion (8%) for the excess.

NAME	INTAKE		24-HOUR EXCRETION		24-HOUR BALANCE	
	g Protein (N x 6.25)	mmol Leucine	g Protein/d (N x 6.25)	mmol Leu/d	g Protein/d	mmol Leu/d
A. OBESE						
FB	80.9	60.4	89.7	65.8	- 8.8	- 5.4
SH	71.4	53.2	73.3	54.4	- 1.9	- 1.2
DH	69.9	52.1	84.5	61.0	-14.6	- 8.9
MH	71.4	53.3	85.4	61.8	-14.0	- 8.5
ML	73.8	51.8	-	-	-	-
MT	78.6	58.8	85.2	62.8	- 6.6	- 4.0
MW	72.3	52.4	85.3	60.3	-13.0	- 7.9
TD	66.9	48.1	88.9	61.5	-22.0	-13.4
EK	68.4	52.6	71.9	54.7	- 3.5	- 2.1
RO	72.3	53.5	74.3	54.7	- 2.0	- 1.2
MEAN	72.6	53.6	82.1	59.7	- 9.6	- 5.8
± S.D.	±4.3	±3.5	±6.9	±4.1	± 6.8	± 4.2
B. CONTROL						
MG	65.6	48.8	67.3	49.8	- 1.7	- 1.0
JS	65.6	48.8	84.6	60.4	-19.0	-11.6
JCW	65.8	46.3	92.9	62.8	-27.1	-16.5
RWS	72.3	52.4	101.3	70.1	-29.0	-17.7
JKG	66.7	48.1	76.0	53.8	- 9.3	- 5.7
MEAN	67.2	48.8	84.4	59.4	-17.2	-10.5
± S.D.	±2.9	±2.2	±13.4	±7.9	±11.7	± 7.1

APPENDIX 3.1. Protein turnover in 4 non-obese 'Control' Group subjects measured over 24 hours, by continuous intravenous infusion of (¹⁵N)glycine, using urinary ammonia as end-product.

A. FEEDING 12 HOURS

NAME	Q	Z	B	E
g Protein per 12 Hours				
MG	130.9	97.3	65.3	33.6
JS	120.0	77.8	54.4	42.1
JCW	171.0	124.2	108.2	46.8
RWS	117.7	63.7	45.4	54.0
MEAN	134.9	90.8	68.3	44.1
± S.D.	±24.7	±26	±28	±8.5

B. FASTING 12 HOURS

NAME	Q	Z	B	E
g Protein per 12 Hours				
MG	58.0	24.5	58.0	33.6
JS	64.7	22.6	64.7	42.1
JCW	94.7	47.8	94.7	46.8
RWS	85.1	31.2	85.1	54.0
MEAN	75.6	31.5	75.6	44.1
± S.D.	±17	±11	±17	±8.6

APPENDIX 3.2. Oral single dose (^{15}N)glycine/ NH_3 end-product method measurements of protein turnover, in 6 obese patients on 'Normal' Diet, and 3 of these patients on 'Diet O'. Measurements were made in Feeding Phase only.

A. NORMAL DIET (Feeding 12 Hours)

NAME	Q	Z	B	E
g Protein per 12 Hours				
MK	277.7	243.7	210.7	34.0
MW	116.8	80.8	44.8	36.0
RP	131.8	95.9	59.8	36.0
ES	203.9	163.2	128.9	40.7
TD	146.3	106.0	79.3	40.3
DH	139.4	102.0	66.3	37.5
MEAN	169.3	131.9	98.3	37.4
± S.D.	±60.9	±61.5	±62	±3

B. DIET O (Feeding 12 Hours)

NAME	Q	Z	B	E
g Protein per 12 Hours				
MK	69.9	58.1	69.9	11.8
TD	74.8	67.3	74.8	7.5
DR	67.2	57.1	67.2	10.1
MEAN	70.6	60.8	70.6	9.8
± S.D.	±3.9	±5.6	±3.9	±2.2

APPENDIX 4.1. Protein turnover rates in 4 obese women (DIET O-DIURNAL GROUP), measured over 24 hours by (1-¹⁴C)leucine infusion, initially on 'Normal' Diet, and then 3 weeks later on Diet O (see Appendix 4.2).

A. NORMAL DIET

FEEDING PHASE

NAME	Q	Z	B	E	I
g Protein per 12 Hours					
MW	174.7	140.5	88.7	34.2	72.3
TD	164.6	117.1	85.8	47.5	66.9
EK	180.2	134.1	94.1	46.2	68.4
RO	127.1	90.3	39.4	36.9	72.3
MEAN	161.7	120.5	77.0	41.2	70.0
± S.D.	±24	±22	±25	±7	±2.8

FASTING PHASE

NAME	Q	Z	B	E	I
g Protein per 12 Hours					
MW	150.8	125.5	150.8	25.3	0
TD	84.8	70.2	84.8	14.6	0
EK	106.7	86.7	106.7	20.0	0
RO	105.5	85.5	105.5	19.9	0
MEAN	112.0	92.0	112.0	20.0	0
± S.D.	±28	±24	±28	±4	

APPENDIX 4.2. Protein turnover rates measured by (1-¹⁴C)leucine infusion over 36 hours, in 4 obese women (DIET O-DIURNAL GROUP) after 3 weeks on Diet O. After the Feeding and Fasting Phases of Diet O, Diet P (500 Kcal/50 g Protein) was introduced (3 patients).

A. DIET O

FEEDING PHASE

NAME	Q	Z	E	I
		g Pro	Hours	
MW	82.0	76.1	.0	5.9
TD	83.4	79.6	88.4	8.8
EK	82.5	73.8	82.5	8.7
RO	63.9	57.9	63.9	6.0
MEAN	79.2	71.9	79.2	7.4
± S.D.	±11	±10	±11	±2

FASTING PHASE

NAME	Q	Z	B	E	I
		g Protein per 12 Hours			
MW	79.2	73.1	79.2	6.2	0
TD	88.1	81.3	88.1	6.8	0
EK	74.9	68.2	74.9	6.7	0
RO	64.5	58.8	64.5	5.7	0
MEAN	76.7	70.4	76.7	6.4	0
± S.D.	±10	±9	±10	±0.5	

B. DIET P (PROTEIN REFEEDING)

FEEDING PHASE

NAME	Q	Z	B	E	I
		g Protein per 12 Hours			
MW	111.1	89.2	50.7	21.9	48.1
EK	93.5	68.1	33.1	25.4	48.1
RO	89.1	65.0	28.7	24.1	48.1
MEAN	97.9	74.1	37.5	23.8	48.1
± S.D.	±12	±13	±11	±2	

APPENDIX 5.1. Amino Acid composition of milk used to compute O_2 uptakes and CO_2 outputs for amino acid oxidation.

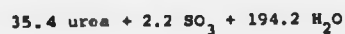
		mg/g N (in milk)	mmoles AA/g N (in milk)
Leu	$C_6H_{13}NO_2$	640	4.9
Pro	$C_5H_9NO_2$	590	5.1
Lys	$C_6H_{14}N_2O_2$	510	3.5
Val	$C_5H_{11}NO_2$	460	3.9
Glu/Glu-NH ₂	$C_5H_9NO_4/C_5H_{10}N_2O_3$	1440	9.8
Asp/Asp-NH ₂	$C_4H_7NO_4/C_4H_8N_2O_3$	530	4.0
Ser	$C_3H_7NO_3$	370	3.5
Ileu	$C_6H_{13}NO_2$	350	2.7
Phe	$C_9H_{11}NO_2$	340	2.1
Thr	$C_4H_9NO_3$	310	2.6
Tyr	$C_9H_{11}NO_3$	280	1.5
Arg	$C_6H_{14}N_4O_2$	250	1.4
Ala	$C_3H_7NO_2$	240	2.7
His	$C_6H_9N_3O_2$	190	1.2
Met	$C_5H_{11}NO_2S$	180	1.2
Gly	$C_2H_5NO_2$	140	1.9
Trp	$C_{11}H_{12}N_2O_2$	90	0.4
Cys	$C_6H_{12}N_2O_4S_2$	60	0.5

Thus for each g of N in milk:



286.96 O_2
millimoles

235.4 CO_2
millimoles

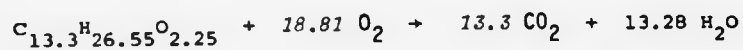


$$R.Q. = 0.823$$

APPENDIX 5.2. Fatty Acid composition and Glycerol in milk-based diets used to compute O_2 uptake and CO_2 outputs for oxidation. It is assumed for calculation purposes that milk fat is only in triglyceride form.

Oleate	27.5 %	$C_{18}H_{34}O_2$
Palmitate	25.5 %	$C_{16}H_{32}O_2$
Stearate	11.0 %	$C_{18}H_{36}O_2$
Myristate	11.0 %	$C_{14}H_{28}O_2$
Glycerol	25.0 %	$C_3H_8O_3$

Thus for 1 mole of 'Mean Triglyceride':



$$\underline{R.Q. = 0.707}$$

APPENDIX 5.3. Conversion of the carbohydrate content of Diet O into available monosaccharide. The factors are calculated by allowing for hydration of the bonds linking sugar units.

1 g Starch \longrightarrow 1.1 g Glucose

1 g Disaccharide (eg. lactose) \longrightarrow 1.05 g Glucose

DIET O 'HYCAL' - Contains 106.24 g mixed carbohydrate/bottle.

	Carbohydrate (g)	Conversion Factor	Glucose Equivalents (g)
Glucose	20.57	1	20.57
Maltose	15.11	1.053	15.91
Trisaccharides	12.54	1.071	13.43
Tetrasaccharides	10.56	1.081	11.42
Pentasaccharides	8.88	1.087	9.65
Polysaccharides	38.58	1.1	42.44
TOTAL	106.24 g CHO		113.42 g Glucose

APPENDIX 5.4. The Stoichiometry of fuel metabolism in Table 8.4.

